

## Heterologous Expression of Human IL-29 (IFN- $\lambda$ 1) in Iranian *Lizard Leishmania*

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**Background:** Interferons with different functions such as antiviral, antiproliferative and immunomodulatory actions are effective medications for a number of diseases. One of these new interferons (IFNs) is Interleukin-29 (IL-29) belongs to the family of IFN- $\lambda$  has antiviral activity and its potent in accompanying with IFN- $\alpha$  in treatment of HCV infection has been evaluated (clinical trial phase II). Recombinant IL-29 has been previously produced in multiple expression systems but in this study we cloned and expressed this protein in an Iranian *Lizard leishmania* (L.L.L) for the first time.

**Objectives:** The Main objective of this research was to evaluate expression of functional human IL-29 in domestic *Lizard leishmania* as an alternative eukaryotic expression system.

**Materials and Methods:** The IL-29 expression cassette was constructed into a pLEXY vector. The *leishmania* cells were transfected by electroporation. After selection of transfectants, the protein expression was evaluated at RNA and protein levels.

**Results:** Expression cassette was successfully transfected to *leishmania* cells and expression of recombinant IL-29 was proved by RT-PCR and western blotting. Purified protein showed 20% activity compared to standard protein. Enzymatic removal of N-glycan resulted to the shift of protein mobility on SDS-PAGE.

**Conclusions:** Easy handling and culture of this eukaryotic host and mammalian cell like posttranslational modifications are the main advantages of this expression host, but the expression yield of this protein is very low and it seems to be not economic for large-scale production.

**Keywords:** Interferons; Interleukin-29; Recombinant; *Leishmania*

### 1. Background

Interferons (IFNs) are the most important proteins among the cytokines, which have the variety of roles such as antiviral, antiproliferation and immunomodulation activities. Human Interferons divided into three types as: type I containing IFN  $\alpha$  (13 subtypes), IFN  $\beta$ ,  $\kappa$ ,  $\epsilon$ ,  $\omega$ ,  $\tau$ ,  $\zeta$ ,  $\delta$  and  $\nu$  subtypes (1). Type II of interferon family consists of a single member IFN  $\gamma$ . All of the interferons type I interact with the same type of receptors on the cell surface consisted of the heterodimeric complex of IFN- $\alpha$  $\beta$ R (2, 3). Type I IFNs are responsible for inducing transcription of a large cluster of genes which play a role in host resistance to viral infections, as well as activation of key components in innate and adaptive immune systems including antigen presentation and production of cytokines involved in activation of T cells, B cells, and natural killer cells. The class I IFNs, IFN- $\alpha$  and IFN- $\beta$ , are predominantly immunomodulatory and antiviral factors, whereas IFN- $\gamma$  has a

greater antiproliferative effect. IFN- $\alpha$  is, a family of at least 16 related peptides- 18 - 20 KDa in size- coded by the genes located on chromosome 9 with 85% homology between different members of the group (4). Type III of interferon has three IFN- $\lambda$  (lambda) molecules called IFN- $\lambda$ 1, IFN- $\lambda$ 2 and IFN- $\lambda$ 3 (also called IL29, IL28A and IL28B respectively) (2, 3). These proteins encoded by 3 different genes located on chromosome 19. At the amino acid level IFN- $\lambda$ 2 and - $\lambda$ 3 are highly similar with 96% sequence identity while IFN- $\lambda$ 1 shares approximately 81 % sequence identity with IFN- $\lambda$ 2 and - $\lambda$ 3 (5). After cleavage of the predicted 25 (IL-28A, IL-28B) or 19 (IL-29) amino acid-long signal peptides, the mature polypeptides of IL-28A and IL-28B comprised of 175 amino acids (6). There is no potential N-glycosylation and only one possible O-glycosylation site in IL-28A and IL-28B were observed. The IL-29 gene encodes a mature, secreted IL-29 protein of 181 amino acids, which possess one potential N-glycosylation site and its 3-D structure is comprised of a monomeric  $\alpha$ -helical protein, with

#### Implication for health policy/practice/research/medical education:

The evaluation of an alternative easy handling and cheap eukaryotic expression system for IL29 production as a possible future medication.

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topological similarity to IL-10 and other members of the IL-10 family of cytokines. IFN- $\lambda$ s represented an evolutionary link between IL-10 and type I IFNs. These cytokines are expressed by human peripheral blood mononuclear cells and dendritic cells upon infection with viruses or activation of toll-like receptors (TLRs). The IFN- $\lambda$ s do not bind to the IFN- $\alpha\beta$  receptor, instead they exert their activity through a distinct receptor (6). Interferon lambda family receptor composed of two chains, IL-28R $\alpha$  subunit which is IFN- $\lambda$  specific and also responsible for signal transduction and IL-10R $\beta$  subunit that is common among IL-10, IL-22 and IL-26 (2, 3, 7-9).

IFN- $\lambda$  family members (IL-28A, IL-28B and IL-29), same as type I interferons, after binding to their receptor complex, would activate the signal transduction via activation of JAK/STAT pathway that finally leads to the target gene regulation by STAT molecules. These interferon stimulated genes (ISGs) encoding proteins such as Mx1, OAS or IFIT, which mediate the antiviral effects of IFN (10). Type III IFN was also shown to display antiproliferative and immunomodulatory properties similar to IFN type I members (8, 11-14). Recombinant IFN- $\lambda$ 1 (IL-29) was previously produced in *Escherichia coli*, human A549 cell, *Pichia pastoris*, mouse NSO cell and CHO cell line (15-18). *Leishmania* sp. Protozoa from the family of *Trypanosomatidae*, is another expression system with some unique features such as RNA editing, arrangement of genes in tandem arrays, polycistronic transcription followed by trans splicing, and regulation of gene expression almost exclusively at the post-transcriptional level, high growth rate and easy handling like *E. coli* and yeast expression systems (19-21). In this study we selected Iranian *Lizard leishmania* (I.L.L.) for expression of recombinant human IL-29 (IFN- $\lambda$ 1) (22).

## 2. Objectives

This species of *Lizard leishmania* was isolated in Iran which its potency to express the coagulation factor VII was evaluated by Mirzaahmadi et al. (21). The main objective of this research was the evaluation of this host capability in expression of functional and glycosylated human IL-29 as an alternative host for eukaryotic expression systems.

## 3. Materials and Methods

### 3.1. Cultivation of Iranian Lizard Leishmania (I.L.L.)

Iranian *Lizard Leishmania* I.L.L (22) was cultivated at 26°C in RPMI 1640 (GIBCO, UK) containing 5% fetal bovine serum, 100 units.mL<sup>-1</sup> penicillin and 100  $\mu$ g.mL<sup>-1</sup> streptomycin (GIBCO, Pen-Strep15140), hygromycin (SIGMA, 50  $\mu$ g/mL for selection of recombinant clones). *leishmania* maintaining culture was maintained at 26°C with diluting suspensions in 5-10 folds into fresh medium twice per week. For expression studies I.L.L was grown using a ro-

tating incubator (140 rpm) and harvested after 48 hours.

### 3.2. Cloning of IL-29 (IFN- $\lambda$ 1) Gene Fragment into Iranian Lizard Leishmania (I.L.L.)

The human IL-29 gene sequence (Gen Bank accession number AY336716) was used as a template to synthesis the gene by adding *Sal I* and *Kpn I* restriction sites at up and downstream. Synthesized IL-29 gene in the pGH vector (Nedayefan, Iran) was amplified using flanking M13 primers (Table 1) and then digested with *Sal I* and *Kpn I* restriction enzymes (Ferments, Lithuania) to produce a 558 bp gene fragment which then cloned in the pLEXSY-hyg2 plasmid (EGE-232, Jena Bioscience, Germany). In-silico prediction of Cleavage site for signal peptide (L. Mexicana secreted acid phosphatase LMSAP1) linked to IL-29 gene was performed by online Signal program. Resulting construct encoding the IL-29 fused to the C-terminal His 6 tag and N-terminal signal peptide of pLEXSY-hyg2 was transformed into *E. coli* XL1-Blue (stratagene). Desired recombinant clone was selected by colony PCR using P1442 and A264 primers (Table 1) flanking the multiple cloning site of pLEXSY-hyg2 plasmid and then confirmed by digestion with *Ppuml* enzyme which has a restriction site on IL-29 gene and caused to produce the linear form of a plasmid. At the final step and before transfection, IL-29 gene segment was sequenced using P1442 and A264 primers.

### 3.3. Transfection of I.L.L.

The final construct (pLEXSY-hyg2-IL-29) was purified by Plasmid Miniprep Kit (Bioneer, Korea) and linearized by *Swa I* (Ferments, Lithuania) and the pure expression cassette was isolated by QIAquick Gel Extraction Kit (QIAGEN, USA). 5  $\mu$ g of DNA used for transfection of I.L.L. by electroporation (gene pulser Xcell, Biorad) (23, 24). Stable transfectants were selected on RPMI media containing 25  $\mu$ g.mL<sup>-1</sup> hygromycin B after one week and a stringent selection was continued by increasing the concentration of hygromycin up to 100  $\mu$ g.mL<sup>-1</sup> for another week. For investigating evaluation of the expression cassette into the *ssu* locus of I.L.L., 1 mL aliquot of culture was subjected to genomic DNA extraction and diagnostic PCR was performed using IL-29 reverse primer and F3001 forward primer (Table 1) which located in the *leishmania ssu* gene (annealing temperature 62 °C) (25).

### 3.4. Expression of Recombinant IL-29

Expression of the integrated IL-29 gene was evaluated by RT-PCR and western blot tests. Selected positive transfectants of I.L.L. were grown for 48h incessantly (140rpm). When cell density reached to about 1x10<sup>8</sup> cells, it was centrifuged for 5 min at 3000. The total RNA were extracted by RNeasy mini kit (QIAGEN) and cDNA synthesis was conducted by random hexamers and PCR was carried out with IL-29 specific primers (Table 1, annealing 65 °C). To

analysis the protein secretion, 16 mL of filtered culture supernatant was mixed with 4 mL 50% ice-cold trichloroacetic acid and incubated for 30 minutes on ice then centrifuged for 15 minutes at 12000 rpm. Supernatant was removed completely and then the pellet was resuspended in 1 mL acetone and centrifuged for 15 minutes at 13000 g and 4 °C. The pellet was dried and resuspended in gel loading buffer for SDS-PAGE and western blot analysis.

As mentioned above, for western blot analysis the cultured cells electrophoresed in 12% gel, then transferred to nitrocellulose membrane. The membrane was blocked for 1 hour at room temperature in TBS buffer (20 mM Tris and 150 mM NaCl, pH 7.6) containing 3% nonfat dry milk followed by a 2-hour incubation in the same buffer containing 1000 fold dilution of rabbit anti-IL-29 antibody (Abcam, UK). After 3 - 5 washings with TBST (TBS + 0.1% tween - 20) the membrane was incubated again for 2 h at room temperature in TBS containing 10,000-fold dilution of alkaline phosphatase-conjugated goat anti-rabbit antibody (Abcam, UK), followed by rinsing 5 times in TBST. Color development was achieved by adding alkaline phosphatase substrate (NBT and BCIP) to presoaked membrane in alkaline phosphatase buffer (100 mM diethanolamine, 100 mM NaCl, 5 mM MgCl<sub>2</sub>, pH 9.5).

**Table 1.** Oligonucleotides sequence Used in this Study

| Name           | Sequence (5'to 3')           |
|----------------|------------------------------|
| <b>M13 F</b>   | GTAAACGACGGCCAGTG            |
| <b>M13R</b>    | GGAAACAGCTATGACCATG          |
| <b>IL-29 F</b> | GTCGACGCTGGCCCTGTCCCACTTC    |
| <b>IL-29 R</b> | GGTACCGGTGGACTCAGGGTGGGTTG   |
| <b>P 1442</b>  | CCGACTGCAACAAGGTGTAG         |
| <b>A 264</b>   | CATCTATAGAGAAGTACACGTA AAAAG |
| <b>F 3001</b>  | GATCTGGTTGATTCTGCCAGTAG      |

### 3.5. Purification of Recombinant IL-29

Purification of recombinant IL-29 fused to His-tag was performed on I.L.L. Host cells were incubated for 48h at 140 rpm. They were centrifuged for 10 min at 3000 and washed with PBS. Cleared cell lysates was prepared by adding 5 mL buffer B (Urea, NaH<sub>2</sub>PO<sub>4</sub>, Tris-Cl, pH 8.0) to cell pellets. Cell lysis was performed by gently vortexing at room temperature. When solution became translucent, cell debris was removed by centrifugation at for 20 minutes. 1 mL of the Ni-NTA His-bind resin (Novagen, Merck) was added to lysate and mixed gently by shaking for 60 minutes at room temperature. Lysate -resin mixture was loaded into an empty column and after flow through collection, the resin was washed with 2 X 4 mL buffer C (urea, NaH<sub>2</sub>PO<sub>4</sub>, Tris-Cl, pH 6.3) followed by two step elution: first with 2 mL buffer D (urea, NaH<sub>2</sub>PO<sub>4</sub>, Tris-Cl, pH 5.9) and finally with 2 mL buffer E (urea, NaH<sub>2</sub>PO<sub>4</sub>, Tris-Cl,

pH 4.5). Final eluted proteins were pooled and concentrated by ultrafiltration using Amicon Ultra-15 units with a cut-off of 10 kDa (Millipore, Germany).

### 3.6. Antiviral Assay

The antiviral activity of purified recombinant IL-29 was measured as previously described (26, 27). Briefly trypsinized A549 cell line was re-suspended as single-cell suspensions then seeded in 96 - well microtiter plates at  $3.1 \times 10^4$  per well and incubated at 37 °C in 5% CO<sub>2</sub> for 16 h. The serial 10 fold dilutions of purified recombinant IL-29 and recombinant standard IL-29 (R&D systems, USA) were prepared with culture medium and then transferred to plate in duplicate rows. After 24 h incubation, the culture medium was drained and replaced by medium containing *Encephalomyocarditis virus* (EMCV) in all wells, except control cells with multiplicity of infection (m.o.i) of 10 plaque forming units per cell (pfu.cell<sup>-1</sup>). Plates were incubated for 24 h at 37 °C, then cells were washed with PBS and stained with 0.05% amido-blue black in 0.1 M sodium acetate buffer for 0.5 h at room temperature. After fixation with 4% formalin acetate, cells were washed, dried and then absorbed color was released by 0.1 M sodium hydroxide. Finally absorbance read at 630 nm. Dose related responses were plotted as absorbance versus cytokine concentration.

### 3.7. Glycosylation Study

There is a potential site of N-glycosylation in the IL-29 structure. Glycan enzymatic cleavage of the polypeptide using glycosidases is one of the techniques that do not require special devices. Using this approach, N-linked oligosaccharides are cleaved from the polypeptide by *N-glycosidase F* (PNGase F, Sigma). Electrophoretic mobility of protein before and after treatment with glycosidase was compared.

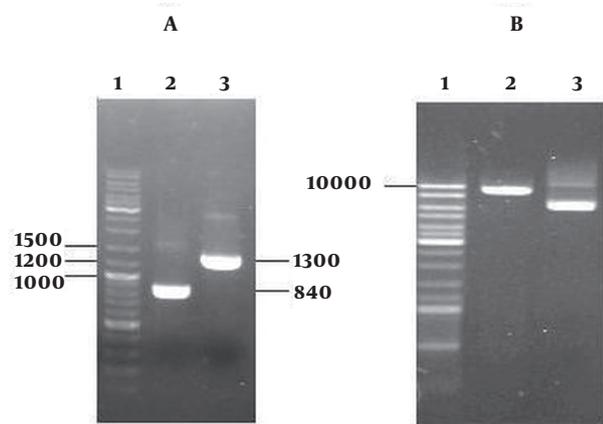
## 4. Results

### 4.1. Construction of Recombinant pLEXSY - hyg 2 - IL-29 Plasmid

In order to obtain a high concentration of IL-29 gene for the ligation reaction, PCR reaction was performed using flanking M13 primers on recombinant pGH-IL-29 construct. PCR product digested with *KpnI* and *Sall* restriction enzymes. This gene fragment was inserted into pLEXSY-hyg 2 expression vector. In-silico SignalP assigns a probability of ~97% for cleavage of signal peptide between the 23<sup>rd</sup> and 24<sup>th</sup> amino acids. Desired clone of transformed *E. coli* was selected as described in materials and methods (2.2) which had a PCR product of about 840 bp vs. clones with 1300 bp-product which contained pLEXSY vectors with re-ligated stuffer instead of desired insert (Figure

1 A). Plasmid extracted from this clone also subjected to further confirmation by digesting with *Ppu*I enzyme (Figure 1 B). Sequencing results revealed that IL-29 gene segment was accurately amplified and located in the correct position.

**Figure 1.** Selection and Confirmation of Desired Transformed *E.coli* by Colony PCR and Restriction Mapping.



Colony PCR using P1442 and A264 primers flanking the multiple cloning site of pLEXSY-hyg2 plasmid was performed. (A) Lane 1, DNA size marker; lane 2, desired colony (840 bp product) resulted from correct ligation of IL-29 gene in the corresponding site, and lane 3, colony with re-ligation of the stuffer segment of vector with the 1300 bp product. (B) Plasmid extracted from the desired colony for more confirmation, digested by *Ppu*I restriction enzyme which has two recognition sites in the IL-29 gene. Lane 1, DNA size marker; lane 2, linearized plasmid and lane 3, uncut plasmid DNA.

#### 4.2. Transfection and Selection of Recombinant

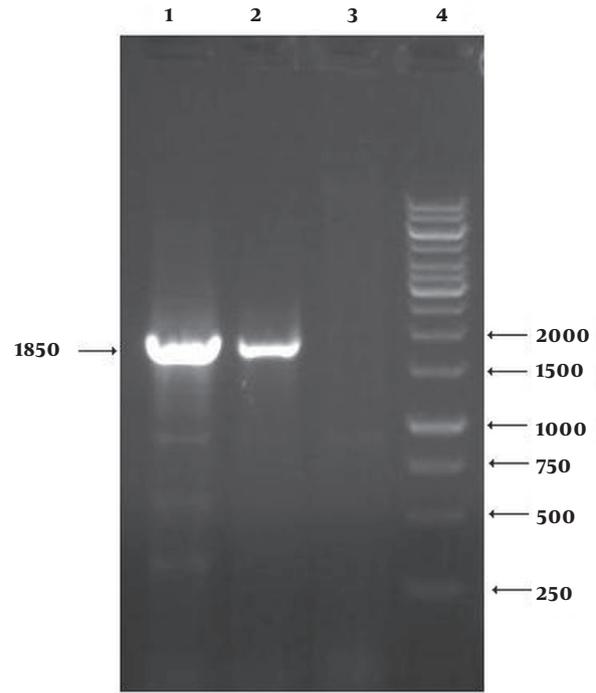
##### I.L.L. Cells

Purified pLEXSY - hyg2 - IL-29 was digested by *Swa* I and the needless parts (*ori* and *bla*) were removed and a 5800 bp pure expression cassette was eluted from the gel to further transfection. Stable transfectants in 100  $\mu$ g.mL<sup>-1</sup> of hygromycin was subjected to DNA extraction and checked for appropriate recombination of interest IL-29 gene in *leishmania* chromosomal 18 srRNA locus (*ssu*) by PCR using F3001 and IL-29 reverse primers (Figure 2). 1850 bp PCR product reveals the successful integration of the expressing cassette into *leishmania ssu* gene.

#### 4.3. Expression Analysis of Recombinant IL-29

Expression of integrated recombinant IL-29 gene was evaluated at both RNA and protein level. RT-PCR results revealed that expected-size IL-29 was transcript and expressed (Figure 3). The presence of IL-29 protein was confirmed by western blot analysis on cell lysate and culture supernatant using specific antibody for IL-29 protein.

Expected major encoded protein include signal peptide and His-tag is about 23 kDa but 30 kDa and greater than 40 kDa isoforms were also observed in cell lysate but not in culture supernatant (Figure 4).



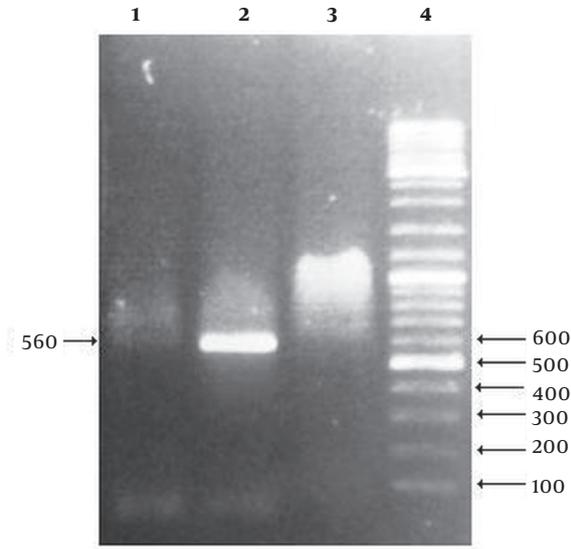
**Figure 2.** Confirmation of Genomic Integration by PCR. Diagnostic PCR using one primer hybridizing within the expression cassette (reverse primer of IL-29) and one primer hybridizing to the *ssu* sequence not present on the plasmid (F3001). Lane1 and 2, different transfectants; lane3, non transfected wild type control, without any PCR product and lane 4, DNA size marker.

#### 4.4. Purification and Antiviral Assay of Recombinant IL-29

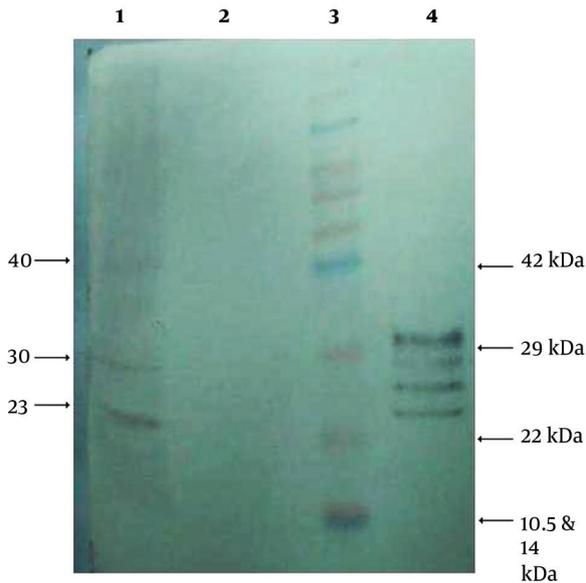
Purification of IL-29 was carried out by Ni - NTA His-bind resin under denaturing conditions and preparation with two major proteins with 23 and 30 kDa molecular weight in SDS - PAGE, under reducing conditions. The final protein yield was 0.075 mg.L<sup>-1</sup> of the suspension culture. Dose-response data obtained from antiviral assay were plotted for purified IL-29 (sample) and standard form (control) (R&D systems, USA). The sample ED50 reached to 25 ng.mL<sup>-1</sup> which was 20% of standard activity with ED50 of about 5 ng.mL<sup>-1</sup> (Figure 5).

#### 4.5. Glycosylation

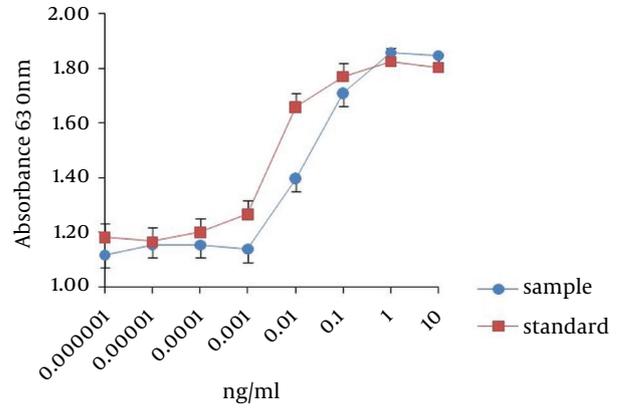
The mobility of recombinant protein on SDS - PAGE after PNGase F treatment showed a small shift comparing with untreated protein, which may be due to enzymatic removal of N-glycans (Figure 6).



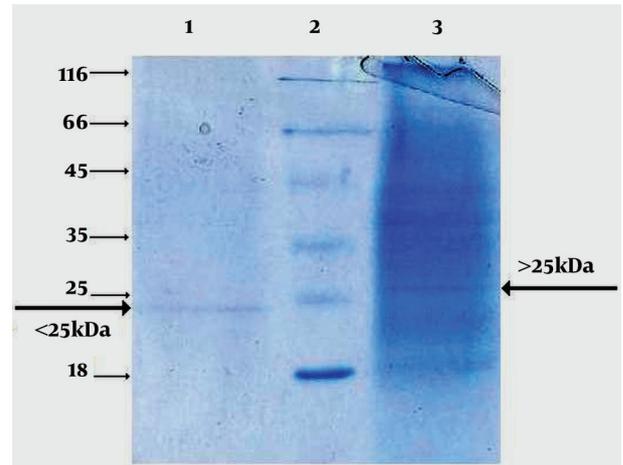
**Figure 3.** RT - PCR Analysis of Gene Expression. Total RNA was extracted and cDNA was synthesized and PCR using IL-29 forward and reverse primers was performed. lane1, non transfected control; Lane2, transfected cells; lane3, extracted RNA as template and lane 4, DNA size marker.



**Figure 4.** Western Blot Analysis of Recombinant Expressed Protein. Selected Transfectants were grown and cell lysate and supernatants were being evaluated for protein expression. Lane 1, cell lysate; lane 2 culture supernatant precipitated by TCA for the presence of secreted protein; lane3, pre stained ladder and lane 4, standard IL-29.



**Figure 5.** Bioassay of Purified Recombinant IL-29. Dose response relationship between cytopathic effect reduction (CPE) and cytokine concentration in the human A549 lung carcinoma cell line-based antiviral assay (AVA). Error bars represent the standard deviations (SDs) of results for duplicate dilutions.



**Figure 6.** Enzymatic Deglycosylation. Expressed recombinant protein was evaluated for N-glycosylation by PNGase F treatment. Electrophoretic mobility of purified recombinant protein treated with enzyme (lane1), and without enzymatic treatment (lane3). Lane 2, protein ladder.

## 5. Discussion

In the last decade, many publications about IL-29 and other interferon lambda family were presented. Although the combination of ribavirin and IFN- $\alpha$ 2 has been established as the standard treatment for patients with chronic HCV infection but the hematologic and neurologic side effects associated with this therapy are the main problems. Since IFN type III receptors are not expressed on all hematopoietic precursor cells, it has predicted to have fewer side effects (28, 29). For these reasons a phase II clinical trial is ongoing. In addition IL-29 independent-

ly has some antitumor activities and in cooperation with type I IFN used for therapeutic purposes of some kinds of tumors (5, 28, 30).

There are many other proteins were expressed in the lexsy eukaryotic expression system (Jena Bioscience lexsy host P10) or *leishmania tarentolae* (31, 32). In this study we used Iranian *Lizard leishmania* parasite which isolated from *Lizard* species (*Agama caucasica microlepis*) in Iran as an expression system for production of recombinant IL-29 for the first time. The main advantages of the *leishmania* expression system are as follows: (i) low-cost growth condition, (ii) faster growth rate, (iii) human safety, (iv) production of recombinant proteins and (v) posttranslational modification of target proteins with a mammalian - type N-glycosylation pattern and correctly protein folding (33).

The expression cassette constructed in pLEXSY Hyg - 2 vector and inserted in the chromosomal location for constitutive expression. In spite of protein production in the cell lysate we could not detect secreted protein in culture media which may be due to the lower efficiency of the signal peptide of secreted acid phosphatase 1 (SAP1) of *leishmania Mexicana* in I.L.L. Secretion process may be improved by changing of some amino acids in signal peptide specific positions (34).

In this study final yield of protein was considered  $0.075 \text{ mg.L}^{-1}$  which was very low in comparison with other proteins expressed in *leishmania tarentolae* (lexsy host P 10 of Jena bioscience) eukaryotic expression system. I.L.L must be well characterized and its proteolytic enzymes and their activity must be elucidated. On the other hand, bioactivity of the purified protein was 20% of utilizing parallel standard which may be due to the purification denaturing condition and restoration maybe are not followed properly for most of the purified proteins. Enzymatic removal of N-glycan on purified recombinant IL-29 revealed that glycosylation as one of the post translational modifications is done at this expression system as expected in eukaryotic systems.

In conclusion, we have expressed, purified and characterized IL-29 (IFN- $\lambda$ 1) using pLEXSY - hyg 2 - IL-29 vector in a new domestic *leishmania* host for the first time. Easy handling and culture of this eukaryotic host and mammalian cell like posttranslational modifications are the main advantages of this expression system. The protein yield expression of this system is very low and it seems is not economical for large-scale production.

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There is no acknowledgment.

## Authors' Contribution

Amir Hossein Taramchi PhD Student carried out his thesis. The other authors were advised and coworkered in this research.

## Financial Disclosure

We have no conflict of financial interests in this manuscript.

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