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Expression of the Recombinant Precursor and Putative Mature Forms of Human interleukin-37 isoform b (IL-37b) in *E. coli* Expression System

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RESEARCH ARTICLE

Abstract

Background and Objectives: IL-37b is a cytokine that may exist in several forms, including a full-length precursor protein and its putative mature forms (IL-37b cleaved at E21, V46, and K53, respectively). In recent years, the role of IL-37b has been associated with the regulation of inflammation and inflammatory diseases. Previous studies focused on the intracellular activity of the cytokine, while the bioactivities of its variants when introduced in the extracellular environment has been limited and require further investigation. To enable this, the study produced precursor and truncated forms of IL-37b in an *E. coli* expression system.
Methodology: Recombinant proteins of the full-length (FL) and shorter forms (E21, V46, and K53) of IL-37b were produced in IPTG-induced *E. coli* BL21-CodonPlus(DE3)-RIPL strain and subsequently purified using Ni^{2+} NTA affinity, ion exchange, and size exclusion chromatography. The identity of the proteins was confirmed through western blotting and LC-MS.
Results: Findings showed that the masses of the expressed proteins correspond to their respective theoretical masses with 24,134.75 ± 0.04 Da for FL, 21,919.63 ± 0.80 Da for E21, 19,298.57 ± 0.04 Da for V46, and 18,551.21 ± 0.04 Da for K53 at 90-95% purity. This confirms that the correct proteins have been produced and at high purity. Further, the tendency of FL to homodimerize was observed in this study, which may have implications in the extracellular processing and bioactivity of FL.
Conclusion: This study describes the successful expression and purification of recombinant precursor and putative mature forms of IL-37b in *E. coli*, which can be utilized for downstream characterization.

Keywords: *Interleukin-37, mature interleukin-37, IL-37b recombinant expression*

Introduction

Interleukin-37 (IL-37), previously known as IL-1F7, is an anti-inflammatory cytokine associated with the attenuation of inflammation, cancer, autoimmune, and inflammatory diseases [1,2,3]. Previous disease models linked IL-37 with a protective function against ischemic and reperfusion injuries in the heart, brain, and liver [4,5,6]. It also prevents inflammatory injuries due to chemical colitis, concanavalin A-induced hepatitis, LPS-induced shock, and aspergillosis in mice [7,8,9,10]. The anti-inflammatory activity of IL-37 is credited to its suppression of pro-inflammatory cytokines, especially in disease models of psoriasis [3,9,11]. Further, IL-37 expression is correlated with reduction of hepatocancer tumor size and inhibition of human cervical cancer cell proliferation and invasion [12,13]. Meanwhile, lowered IL-37 expression is connected with disease progression in patients with Behcet's disease and intervertebral disc degeneration [14,15]. Moreover, exogenous introduction of IL-37 has reduced inflammation-mediated fatigue in mice and enhanced the immunomodulatory functions of regulatory T cells [16,17]. All these highlight the therapeutic potential of IL-37 for treatment of inflammatory-mediated diseases as well as in boosting immunoregulatory functions.

IL-37 exists in five isoforms a to e, each with different tissue distributions, but the most studied and abundant of these is the b isoform. The isoform IL-37b requires cleavage at its propiece for its activation [18,19]. Caspase-1 is implicated in the processing of IL-37b between D20 and E21, without which there is a reduction of anti-inflammatory activity in mouse macrophage cells [20]. However, caspase-1-

dependent cleavage may not be the only mechanism involved in IL-37 maturation. In fact, another truncated form of IL-37b is secreted by IL-37-transfected human embryonic kidney (HEK) 293 and Chinese hamster ovary (CHO) mammalian cells. This IL-37b form is cleaved between F45 and V46, a non-caspase cleavage site [21]. Furthermore, most of the cleavage sites implicated in IL-1 family maturation are nine residues upstream the A-X-D consensus sequence. If this were to be followed strictly, the predicted maturation form of IL-37b starts at K53. While the IL-37b K53 form has not yet been observed in nature, its IL-37 isoform a counterpart (cleaved at V26-K27) is found to have anti-inflammatory activities. IL-37b K53 and IL-37a K27 have the same amino acid sequence though derived from different IL-37 full-length isoforms, which implies that IL-37b K53 may be worth investigating as an active IL-37b form for future therapies [1,22].

To explore the therapeutic potential of IL-37b, they must be introduced exogenously; however, it is not clear which of these IL-37b forms will best exert their anti-inflammatory activities via the extracellular environment. Thus, this study aimed to produce recombinant IL-37b precursor and putative mature forms (E21, V46, and K53) in an *E. coli* expression system. Future research comparing the bioactivities of IL-37b, as well as studies related to elucidating the structure and signaling mechanisms of the full-length and shortened forms may be conducted using the recombinant proteins produced in this study.

Methodology

Unless otherwise specified, reagents and materials were acquired from Sigma-Aldrich, UK, chromatographic columns were taken from GE Healthcare Life Sciences, USA while cell culture reagents were obtained from Lonza, UK.

Generation of Recombinant Precursor and Putative Mature Forms of Human IL-37b

Full-length (GenBank Accession No. Af251119), E21, V46, and K53 IL-37b DNA fragments were generated using the following forward primers FL: ATG TCC TTT GTG GGG GAG, E21: GAA CCC CAG TGC TGC TTA GAA G, V46: GTT CAC ACA AGT CCA AAG GTG AAG, and K53: GTT CAC ACA AGT CCA AAG GTG AAG, respectively. The reverse primer used for all IL-37b forms was CTA ATC GCT GAC CTC ACT G. After amplification with high fidelity KOD Hot Start DNA polymerase (Novagen, Germany), 3'-dA overhangs were added using GoTaq® DNA polymerase (Promega, USA) at 72°C for 20 min. The gene fragments were then inserted into Champion™ pET SUMO expression vector (Invitrogen, CA, USA) per manufacturer's instructions and incubated at 16°C overnight. Constructs were cloned in DH5α *E. coli* and construct sequences were verified using Sanger sequencing (GATC Biotechnologies, UK).

These constructs were transformed into BL21-CodonPlus (DE3)-RIPL *E. coli* (Agilent Technologies, US) by heat-shock method. Expression was induced using 0.8 mM IPTG in 2X YT broth containing kanamycin (33 μ/ml) and chloramphenicol (33 μ/ml). The culture was incubated overnight at 25°C, 160 rpm. After which, the cells were lysed and the IL-37b proteins were isolated using the HisTrap FF column prepacked with Ni^{2+} Sepharose 6 Fast Flow media. The recombinant proteins were desalted through HiPrep 26/10 Desalting Column Sephadex G-25 using 20 mM Tris-Cl, 150 mM NaCl, 2% glycerol buffer at pH 7.4 for FL, K53, and V46, and pH 8.0 for E21, respectively. These were then concentrated using centrifugal ultrafiltration units MWCO 10,000 Da (Millipore, USA). After, the SUMO tag was removed by adding 25 U SUMO protease (Invitrogen, USA) overnight at 4°C. The native protein was then collected through the flow-through of Ni^{2+} NTA column.

The proteins were then subjected to ion exchange chromatography. For FL and E21, HiTrap Q HP column was equilibrated with 20 mM Tris-Cl, pH 8.0 and the proteins were eluted using a 0-80% gradient of 20 mM Tris-Cl, 1 M NaCl, pH 8.0. For K53 and V46, HiTrap SP HP column was equilibrated with 50 mM phosphate buffer, pH 7.0 and the proteins were eluted using a 0-80% gradient of 50 mM phosphate, 1 M NaCl, pH 7.0 buffer.

The proteins were further purified using HiLoad 26/60 Superdex 75 prep grade gel filtration column and equilibrated with 20 mM Tris, 150 mM NaCl, 2% glycerol at pH 8.0 for E21 and pH 7.4 for FL, V46, and K53. Target proteins were concentrated via centrifugal ultrafiltration units MWCO 10,000 Da. Protein products were analyzed by electrophoresis in polyacrylamide gels at non-reducing conditions. Protein identities were confirmed through western blotting with anti-IL-37 clone 261506 antibodies (R&D Systems, USA), and LC-MS at positive electrospray ionization.

Evaluation of Pro-inflammatory Cytokine Inhibition

THP-1 was grown and maintained in RPMI-1640 media (Gibco, USA) with 10% FBS, 0.5% L-glutamine, 50 U/ml penicillin, and 0.05 mg/ml streptomycin. These were challenged with 100 ng/ml LPS and treated with 100 nM recombinant IL-37bs. The treated culture was incubated for 48 hours at 37°C with 5% CO^2. After incubation, the cell

supernatants were collected and IL-8 concentrations were evaluated in duplicates using the BioLegend Human IL-8 ELISA Max kit (BioLegend, USA) following manufacturer's instructions.

Results

Expression and Purification of the Recombinant IL-37b Proteins

To enable further investigation of IL-37b and its putative mature forms, recombinant production of the proteins was carried out through *E. coli*. SUMO/6xHis-tagged proteins were initially generated from the chosen expression system as shown in Figure 1.

The SDS-PAGE migration of SUMO-fused proteins corresponded to their predicted molecular sizes of ~38 kDa for FL, ~35 kDa for E21, ~33kDa for V46, and ~32 kDa for K53 suggesting the successful production of the four proteins in the soluble fraction. Further, when the SUMO/6x His tag was cleaved, a distinct band was generated for the native FL and E21 proteins. On the other hand, the native V46/ K53 proteins tended to run at the same rate as the SUMO tag due to their comparable molecular sizes of ~19 kDa (V46), ~18.6 kDa (K53), and ~13 kDa (SUMO tag).

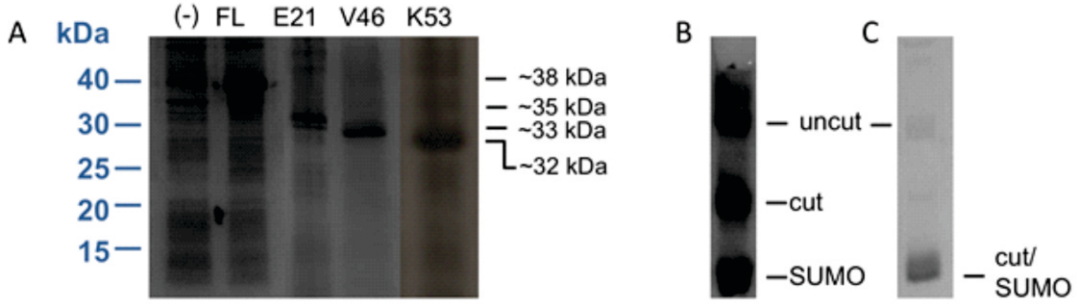

Figure 1. *SUMO/6xHis-tagged IL-37b proteins and tag removal in 17% polyacrylamide gel. (A) SUMO/6xHis-tagged proteins from cell lysates migrated at their predicted molecular sizes of ~38 kDa for FL, ~35 kDa for E21, ~33 kDa for V46, and ~32 kDa for K53; (-) is the uninduced control. (B) Representative profile of SUMO protease digestion of FL/E21 shows the uncut (~35-38 kDa) , native/cut (~22-25 kDa), and removed SUMO tag (~13 kDa). (C) Representative profile of SUMO protease digestion of V46/K53 shows the uncut (~32-33 kDa) and the overlapping migration of the native protein and the SUMO tag.*

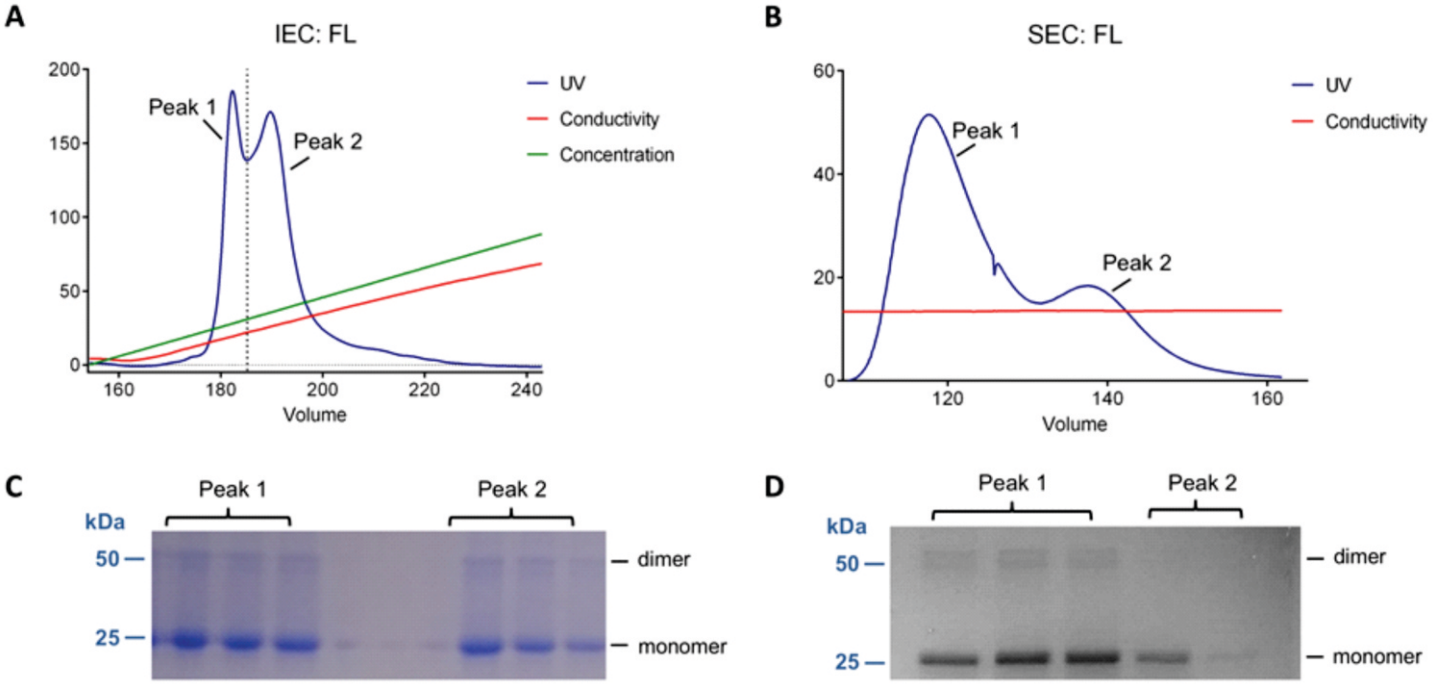

Figure 2. *Purification of the monomeric recombinant FL. (A) IEC and (B) SEC traces show two peaks that is a mixture of ~25-kDa and ~50-kDa proteins. (C) Gel profile of the IEC reflect this mixture of proteins although more of the monomeric FL is eluted in Peak 1. (D) Gel profile of SEC shows that it is more effective in isolating the monomeric FL via Peak 2.*

Further purification via ion exchange (IEC) and size exclusion chromatography (SEC) (Figures 2 and 3) was needed to fully isolate the proteins without the SUMO tags.

From Figure 2, recombinant FL shows a tendency to form dimers as seen from the high absorbances of Peak 1 (Figure 2A and 2B) in both IEC and SEC traces. From the SDS-PAGE profiles, SEC is more effective in the isolation of the FL monomeric form (Figure 2D).

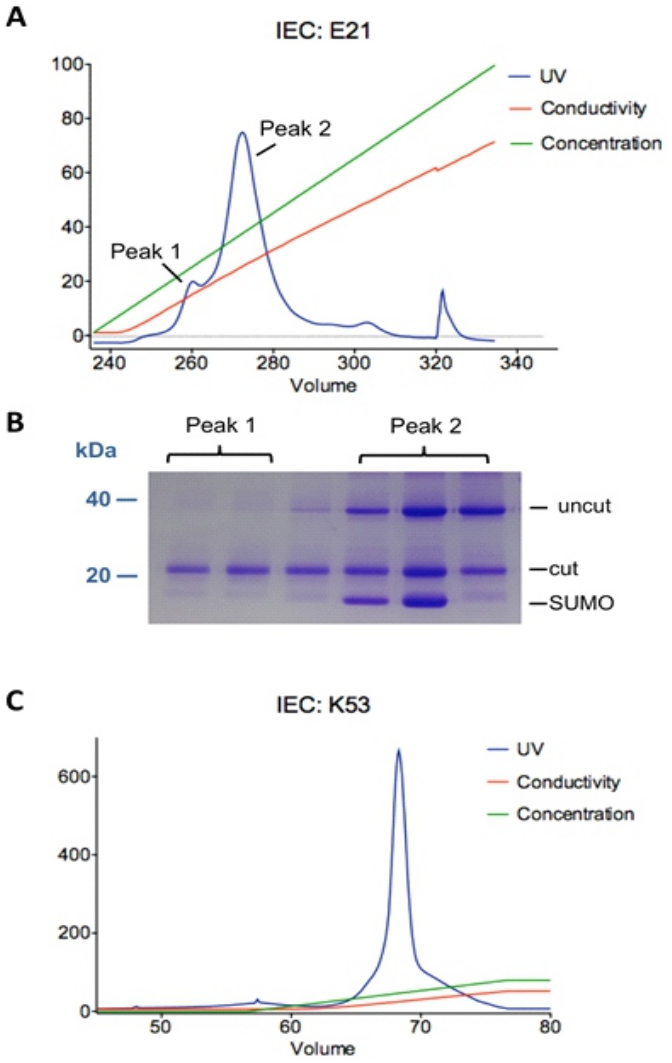

Figure 3. *Purification of the truncated IL-37b forms. (A, B) Peak 1 contains the SUMO-free E21 (~22 kDa), while Peak 2 contains a mixture of the SUMO-tagged E21 (~35 kDa), cut E21 (~22 kDa), and the SUMO tag (~13 kDa). (C) IEC of K53 elutes as a single peak; a similar trace is observed in V46.*

Table 1. *Comparison of the theoretical and experimental masses of recombinant IL-37bs*

Protein	Theoretical Mass (δ)	Experimental Mass (δ)	δ
Full-length (FL)	24 138.5	24 134.75 0.04	3.75
E21	21 924.1	21 919.63 0.80	4.47
V46	19 300.1	19 298.57 0.04	1.53
K53	18 551.2	18 551.21 0.04	0.01

The cut recombinant E21 was isolated in Peak 1 of IEC (Figure 3A and 3B), however, majority of E21 elutes with the SUMO-tagged E21 and the SUMO tag in Peak 2, which compromised the yields of E21. On the other hand, the purification of K53 has been straightforward as it elutes as a single peak in IEC. A single peak was also observed in V46. After IEC, all truncated forms were further purified using SEC and they eluted as single peaks. These purified products were then concentrated and analyzed through western blotting (Figure 4A) and LC-MS (Table 1).

The recombinant proteins without the SUMO tags are predicted to run at ~25 kDa for FL, ~20 kDa for E21, ~19 kDa for V46, and ~18.6 kDa for K53, which are consistent with the immunoblot (Figure 4A) and SDS-PAGE profile (Figure 4B). Further, it was observed that after concentrating the proteins, FL once again formed multimers (predominantly dimers of ~50 kDa).

Table 1 shows that the experimental masses of the proteins corresponded with the theoretical masses of the SUMO-free recombinant proteins derived from ExPASY ProtParam, which confirms the identities of the proteins. Further, through LC-MS, a 48,270.03 ± 3.00 Da protein was detected in the FL sample. This molecular size is twice that of the monomeric FL, which indicates the homodimerization of FL and this has been observed consistently through the western blots and gel profiles.

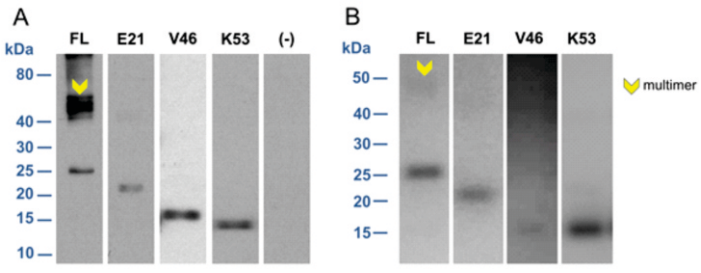

Figure 4. *Isolated recombinant IL-37b proteins. (A) Representative immunoblot probed with anti-IL-37 antibody confirms the isolation of the target proteins; (-) is the cleaved SUMO tag. (B) Representative SDS-PAGE profile of the isolated proteins suggests 90-95% sample purity.*

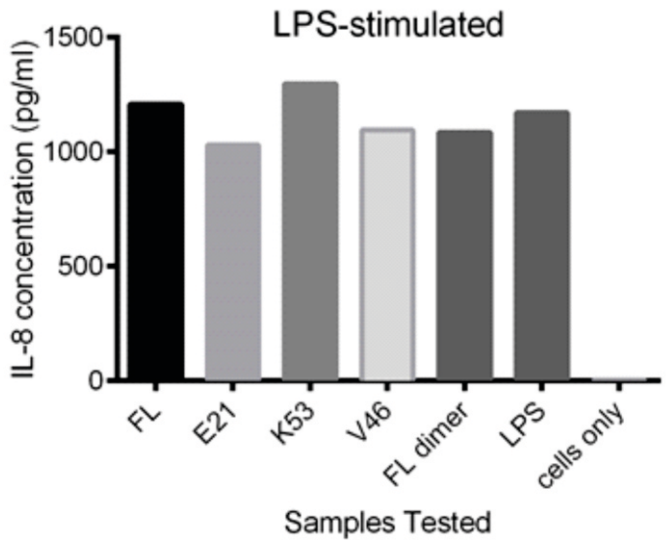

Figure 5. *Non-suppression of IL-8 by 100 nM IL-37b. Based on the mean (n=2) of each treatment, no decrease in THP-1-secreted IL-8 was observed upon treatment of the recombinant IL-37b.*

Assessing the IL-8 Suppression by Recombinant IL-37b Proteins

IL-37 has previously been documented to repress pro-inflammatory cytokines, thus, LPS-stimulated THP-1 cells were treated with the FL and putative mature forms of IL-37b.

Figure 5 demonstrates that IL-37b in nanomolar concentrations was not able to suppress the production of IL-8 by THP-1. Also, there was no significant difference in bioactivities between the precursor and putative mature forms nor between the FL monomer and FL dimer.

Discussion

Validation of Generated Recombinant IL-37b Proteins

The full-length and truncated forms of IL-37b at E21, V46, and K53 were successfully expressed and isolated at a 90-95% purity based on gel profiles. IL-37b molecular weights derived from LC-MS corroborate the theoretical masses of the proteins and agree with previous research involving IL-37b, which confirms the identities of the recombinant proteins.

IL-37b precursor derived from IL-37 transfected RAW cells have been shown to run at approximately 25 kDa, which is comparable to the monomeric FL produced in this study. The same protein sizes were observed for the recombinant IL-37b precursor produced by Gu *et al.* and Ellisdon *et al.*

When comparing the results of this study with that of Gu *et al.*, Gu's group has observed that the recombinant FL is also found in the insoluble fraction of the cell lysate which may translate to loss of protein yield. This problem has not been encountered in this study due to the presence of the SUMO tag which enhances protein solubility, thus ensuring that the proteins produced have good yields and are functional. However, the main challenge in this study is isolating the monomeric FL form as the IL-37 precursor, in general, has a tendency to form dimers or tetramers (to a lesser degree) as previously documented by Kumar *et al.* and Ellisdon *et al.* Although, this may be resolved by keeping the concentrations of FL in nanomolar quantities as the reported dimerization constant for IL-37b precursor is 506 nM. Further, Nold *et al*. observed that endogenous IL-37b from peripheral blood mononuclear cell (PBMC) lysates runs near the 45-kDa mark in SDS-PAGE which emphasizes the dimerization of IL-37b. The function of this IL-37b multimerization has not yet been fully elucidated although it has been connected with the attenuation of the anti-inflammatory activity of IL-37b.

On the other hand, the mature IL-37b E21 derived from transfected RAW cells migrate near the ~20 kDa mark, which is consistent with the observations in this study. E21 was also previously reported to form dimers at lower concentrations than the precursor. However, dimer formation was not observed in the final purified product in this project. This may be due to the loss in protein yields after separation of the native E21 protein from the SUMO tag and the uncut E21. Challenges in the removal of the fusion tag has been previously observed by Kumar *et al.*, but the reason for this is still unknown.

The N-terminus of IL-37b secreted from HEK 293 and CHO was identified to begin at V46. This protein travels at ~27 kDa in SDS-PAGE, which is comprised of the ~8-kDa FLAG tag and the ~19-kDa native V46 protein. In addition, the V46 recombinant protein has been previously observed to migrate between the 15 to 20 kDa range. Both of these agree with the SDS-PAGE migration of the protein produced in this study and the size determined from LC-MS. Moreover, V46 has been documented to dimerize at 294 nM, a lower concentration than the precursor, which suggests a higher tendency to form dimers. It has also been demonstrated to form tetramers at a comparable concentration with the precursor. However, V46 multimerization has not been observed in this study. Further, the V46 processed form was shown to exert a higher reduction in pro-inflammatory cytokines, IL-6, and IL-1b concentrations than the precursor.

This difference in bioactivities between FL and V46 in the extracellular environment is still unexplained.

Regarding IL-37b K53, to date, there are no published data about this particular form. However, since this is identical in amino acid sequence with the recombinant IL-37a K27, it can be assumed that it will have the same molecular size of 18.6 kDa as confirmed by the LC-MS data (Table 1). Further, K53 may also contain the anti-inflammatory properties of K27. In fact, IL-37a K27 has demonstrated suppression of IL-6, IL-1β, and TNFα from LPS-treated models. This implies that the structural components of IL-37b required for immune regulation may be found downstream the sequence, unless different cell types respond to different forms of IL-37b when introduced exogenously.

Most studies tackling IL-37b are focused on the FL and E21 forms, and the anti-inflammatory properties they exert from within the cell. Not much has been said regarding the activity of E21 when applied in the extracellular environment. On the other hand, the precursor and the V46 forms are commonly investigated as secreted proteins and the bioactivities they induce thereafter. However, this view does not take into account if the activation occurred within the cell or outside the cell. Moreover, when comparing the bioactivities of IL-37b-FL-transgenic mice with injected IL-37b precursor, the former is found to be more effective in curbing IL-1β production, which suggests that there is an unknown mechanism that makes intracellularly-derived IL-37b-FL more active. Conversely, it is more advantageous as a therapeutic treatment if IL-37b is introduced exogenously, which means that the most active IL-37b form in the extracellular space has to be determined.

Anti-inflammatory Assay of Exogenous Recombinant IL-37b

To evaluate the anti-inflammatory activity (i.e. suppression of pro-inflammatory cytokine) of the recombinant IL-37bs, these were added in LPS-induced THP-1 cultures. The pro-inflammatory cytokine (IL-8) production of the cells was then measured with ELISA. It was observed that none of the recombinant IL-37bs were able to reduce the level of IL-8 produced. Similarly, a study by Nold, *et al.* demonstrated that silencing of intracellular IL-37b did not affect IL-8 concentrations. These results imply that IL-37b mechanisms may not be linked with IL-8 or that in the extracellular environment, more optimized IL-37b conditions need to be met. Nevertheless, IL-8 is just one of the pro-inflammatory cytokines produced during an immune response, there are other cytokines whose activities may be attenuated by exogenous IL-37b. In fact, it has been previously shown that intracellular IL-37b was able to reduce pro-inflammatory cytokine IL-6, IL-1β, and TNFα concentrations. Going forward, it may be worth investigating if recombinant IL-37b can perform the same activity from the extracellular space. Further, it has been previously reported that IL-37b FL loses its activity in nanomolar and micromolar concentrations and that it functions optimally at picomolar concentrations. From this, it is suggested that the optimal concentration of all IL-37b forms in the extracellular environment be determined through a dose response assay.

Conclusion and Recommendation

This study was able to successfully express and purify the precursor and putative mature forms of IL-37b as confirmed by the immunoblot and LC-MS data. However, IL-8 suppression was not induced in the extracellular space, which suggests that IL-37b anti-inflammatory mechanisms may have a different pathway from IL-8 regulation. Hence, it is suggested that repression by extracellular recombinant IL-37bs be tested on other pro-inflammatory cytokines to evaluate its bioactivity.

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