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Technical note

Expression, purification and characterization of recombinant interleukin-21

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ABSTRACT

Interleukin-21 (IL-21) is a key regulator of the immune system. However, studies of this cytokine have so far been hampered by the limited availability of recombinant protein preparations. Here we describe a method based on refolding of inclusion bodies expressed in *E. coli* by rapid dilution. The method was applied to human and murine IL-21 proteins, which were further purified by affinity chromatography and gel-filtration. The proteins are pure and highly active as determined by endotoxin and cell proliferation assays. The availability of milligram quantities of protein enabled us to generate monoclonal antibody fragments against the cytokine and will aid in further structural, biochemical and physiological analyses.

Interleukin-21, a relatively new member of the common gamma chain signalling class of cytokines, has attracted a great deal of interest in view of its multiple effects on the immune system (Vogelzang and King, 2008; Vogelzang et al., 2008). It shares the gamma chain subunit with a range of other cytokines (including IL-2, IL-4, IL-7, IL-9, IL-13 and IL-15), while specific interactions are mediated through its own receptor (IL-21R) (Parrish-Novak et al., 2000). IL-21 shares sequence identity with IL-15 and, more distantly, with IL-4 and IL-2 (Parrish-Novak et al., 2000). The human cytokine gene is located on the 4q26–27 locus, together with the IL-2 gene (Parrish-Novak et al., 2000). This region has been linked to a range of inflammatory conditions by recent genome-wide association studies (Asano et al., 2006; van Heel et al., 2007; Liu et al., 2008; Festen et al., 2009). In contrast to IL-2, which has been extensively studied, many of the structural, biochemical and physiological properties of IL-21 remain unknown. This is not only due to its more recent discovery, as the ligand of a previously described orphan receptor (Ozaki et al., 2000), but also due to the limited availability of recombinant protein and the prohibitive cost of commercially available preparations.

Although cloning and expression of the IL-21 cytokine has been previously attempted (Asano et al., 2002; Tang et al., 2006; Bondensgaard et al., 2007), we found these studies to be suboptimal for our purposes in several respects. First, none of the mentioned studies reported purification yields, making their scope hard to access. Second, the degree of endotoxin contamination was not reported, which is essential for *in vivo* studies. Finally, no comparison of bioactivity was made with that of commercially available IL-21, despite the fact that bioactivity may have been affected by modifications (such as purification tags).

Here we present methods for the cloning, expression and purification of milligram quantities of endotoxin-free, bioactive interleukin-21 and demonstrate its use in the development of specific affinity reagents.

1. Materials and methods

1.1. Cloning of human and murine IL-21

Regions encoding human IL-21 were amplified by PCR from cDNA using primers 5′-ATGCCCTCGGGGAGAACCTTTACTTCCAGGGTCAAGATCGCCACATGATTAG-3′ and 5′-GTACGTCGACTTATCAGGAATCTTCACTTCCGTG-3′. The cDNA was extracted from human tonsils (kindly provided by C. Ma,

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Garvan Institute). Regions encoding murine IL-21 were amplified by PCR amplified using primers 5′-ATGCCCTCGGGGAGAACCTTTACTTCCAGAGCCATAAATCAAGCCCCCAAGGGC-3′ and 5′-GTACGTCGACTTATTAGGAGAGATGCTGATGAATC-3′. The cDNA was extracted from mouse spleen cells. All studies had been approved by animal and human ethics committees. The amplified regions were cloned into a pUC-based vector using *Ava*I and *Sal*I restriction sites (underlined) encoded in the species-specific PCR primers. In a second step, the IL-21-encoding regions were then amplified by PCR using primers 5′-GTACCATATGGGCCATCACCATCACCACCATGAGAACCTTTACTTCCAG-3′ and 5′-CGCCAGGGTTTTCCCAGTCACGAC-3′. The final PCR products were subcloned into the protein expression vector pET24a (Novagen, Madison, WI, USA) using *Nde*I (underlined) and *Hind*III sites and their sequences confirmed by DNA sequencing.

1.2. Cytoplasmic protein expression

Plasmid DNA was transformed into *E. coli* Rosetta (Novagen) by heat shock transformation (as described in the manufacturer's protocol). Cells were grown overnight at 37 °C from a single colony in 2× TY medium supplemented with 30 μg/ml chloramphenicol and 50 μg/ml kanamycin. The next day, cells were diluted 100-fold into 500 ml of fresh medium (as above) and grown in two litre baffled flasks at 37 °C in an Infors HT shaker at 240 rpm until OD_{600} of 0.5. Protein expression was induced by adding 1 mM IPTG. Cells were grown at 25 °C for 3 h, harvested by centrifugation and stored at −20 °C.

Frozen bacterial pellets were lysed using 30 ml of lysis buffer (50 mM TrisHCl pH 8.0, 100 mM NaCl, 5 mM EDTA and 0.5% Triton-X-100). This was followed by centrifugation at 39800×*g* for 10 min at 4 °C. Pellets were resuspended in 30 ml of lysis buffer and sonicated for 2 min (at a 40% power setting). Resuspension, sonication and centrifugation steps were repeated four times (Triton-X-100 was excluded in the final resuspension step). The resulting pellets (inclusion body pellets) were stored overnight at −20 °C.

1.3. Protein refolding

Inclusion body pellets were resuspended in 20 ml of solubilization buffer (100 mM TrisHCl pH 8.0, 6 M guanidine–HCl solution and 40 mM DTT) to a final protein concentration of 10 mg/ml. The solution was diluted into 2 l of refolding buffer (40 mM TrisHCl pH 7.5, 100 mM L-arginine, 0.5 mM oxidized glutathione, 5 mM reduced glutathione, 20 mM NaCl, 4 mM $MgCl_2$, 1 mM KCl and 4 mM $CaCl_2$) by slowly adding single drops into the stirred refolding buffer (at a rate of 40 ml/h). All steps were carried out at 4 °C and the buffer was stirred overnight at 4 °C. The next day, the refolding buffer was filtered through a 0.2 μm filter and adjusted to pH 7.5 with 5 M HCl solution. Cystine was added to the buffer to a final concentration of 1.6 mM.

1.4. Protein purification

In a first chromatography step, refolded proteins were bound to a Ni-NTA affinity column (5 ml column volume; GE Healthcare, Piscataway, NJ, USA) using a peristaltic pump at a flow rate of 1 ml/min at 4 °C. After loading, the column was washed with 20 ml of wash buffer (20 mM sodium phosphate pH 7.4, 500 mM NaCl and 20 mM imidazole). Proteins were eluted from the column in 25 ml of elution buffer (20 mM sodium phosphate pH 7.4, 500 mM NaCl and 500 mM imidazole). Cleavage of the N-terminal hexa-histidine tags was performed by adding 25 μg/ml of TEV protease (kindly provided by D. Stock, VCCRI Sydney and D. Rhodes, MRC-LMB Cambridge) (Fairall et al., 2001) and incubation at room temperature overnight. Proteins were then concentrated to 500 μl using a 10 kDa Amicon Ultra-15 microconcentrator (Millipore, Billerica, MA, USA). An additional purification step was performed using a Superdex HR75 gel-filtration column (GE Healthcare) and 25 mM phosphate buffer pH 8.5 as running buffer. In order to further reduce endotoxin levels, the proteins were purified by ion exchange on a Capto S column (GE Healthcare). For this, the proteins were bound to the column in 25 mM phosphate buffer (pH 8.5) and eluted using a salt gradient ranging from 0.1 M to 1 M NaCl in 25 mM phosphate buffer (pH 5.5).

1.5. Bioassays

IL-21 bioactivity analysed by measuring proliferation of IL-21-dependent pro-B cell lines (kindly provided by D. Yu, Garvan Institute) (Yu et al., 2007). The cell lines were cultured in RPMI medium (Gibco, Rockville, MA, USA) supplemented with 10% fetal bovine serum, 50 U penicillin, 50 μg streptomycin, 2 mM L-glutamine, 0.1 mM non-essential amino acids (Gibco), 1 mM sodium pyruvate, 10 mM HEPES buffer pH 7.2–7.5, 0.1% β-mercaptoethanol and variable concentrations of IL-21 at 37 °C in 5% CO_2. After three days, thymidine (MP Biomedicals, Irvine, CA, USA) was added to the cultures (1/20 dilution of 40–60 Ci/mmol stock). The next day, incorporation was measured using a Top Count NXT instrument (PerkinElmer, Waltham, MA, USA). Cytokine controls were purchased from a commercial supplier (Peprotech, Rocky Hill, NJ, USA).

1.6. Endotoxin assay

Endotoxin levels were tested using the Limulus Amebocyte Lysate (LAL) assay (Lonza, Basel, Switzerland) following the manufacturer's protocol.

1.7. Selection of anti-IL-21 antibody fragments by phage display

Anti-IL-21 antibodies were selected using phage display technology, essentially as previously described (Lee et al., 2007; Christ et al., 2006). Human IL-21 was used to coat a single row of a 96-well MaxiSorb plate (Nunc, Roskilde, Denmark) at 20 μg/ml in PBS overnight at room temperature. Wells were washed with PBS, blocked with 300 μl of 2% Marvel skim milk powder in PBS (MPBS) for 2 h at room temperature and again washed twice with PBS. Phages (Tomlinson I/J libraries; (Holt et al., 2000)) were added to the wells in MPBS (100 μl per well) and incubated. Wells were then washed three times with PBS supplemented with 0.05% (v/v) Tween-20 and once with PBS. Phages were eluted from the well by adding 100 μl of trypsin protease solution per well and incubating at room temperature for 30 min

(trypsin protease solution: 100 μg/ml in TrisHCl pH 7.5, 137 mM NaCl, 10 mM $MgCl_2$ and 1 mM $CaCl_2$).

The eluted phages were added to 10 ml of *E. coli* TG1 grown to OD_{600} of 0.5 and incubated in a water bath at 37 °C for 60 min. Cells were pelleted by centrifugation at 3220×*g* for 5 min, resuspended in 2× TY medium and plated on TYE agar supplemented with 100 μg/ml of ampicillin and 4% glucose. After incubation at 37 °C over night, cells were then scraped from agar plates. From these, phages were produced for subsequent rounds of selection using KM13 helper phage (Kristensen and Winter, 1998). A total of three rounds of selection on human IL-21 were performed, after which single clones were tested for binding to antigen by soluble ELISA. Positive clones were sequenced, expressed and purified by protein A affinity chromatography.

1.8. Biacore analyses

Antigen was immobilized on a CM5 Sensor Chip (Biacore Life Sciences, Uppsala, Sweden) by amine coupling. Serial dilutions of purified scFvs were injected onto the sensor chip surface at a flow rate of 10 μl/min. Measurements were made in HBS-EP buffer on Biacore SPR 2000 system.

2. Results and discussion

2.1. IL-21 cloning, expression and purification

In order to generate expression plasmids for the production of human and murine IL-21, we amplified the corresponding genes by PCR. As templates we used cDNA harvested from lymphoid tissues, which had previously been shown to express the cytokines at high levels (Parrish-Novak et al., 2000). The amplified genes were cloned into a commercially available cytoplasmic expression vector (Novagen pET24a). This vector contains the T7 promoter and encodes an N-terminal hexa-histidine tag for affinity purification. In addition, a TEV site was introduced at residues 9–14 (Fig. 1), allowing removal of the terminal affinity tag through protease cleavage.

Both murine and human IL-21 were expressed at very highly levels (>100 mg/l) from this construct when transformed into *E. coli* and grown in shaking flasks. However, the proteins were almost exclusively found in inclusion bodies and very little was observed in soluble fractions. This was not unexpected as the proteins contain multiple cysteines and may not fold correctly in the reducing environment of the bacterial cytoplasm. Approaches to increase the proportion of soluble protein through the use of N-terminal fusions (maltose binding protein), periplasmic expression or different bacterial strains (Novagen Origami) had only limited success (data not shown).

We therefore focused on refolding the proteins from inclusion bodies. For this, the proteins were denatured in 6 M guanidinium chloride solution and then rapidly diluted into the refolding buffer, by dripping single drops of protein solution into the stirred buffer. The refolding buffer, in which the proteins were stirred overnight, was based on a glutathione redox-shuffle and oxidizing conditions. The proteins were then purified and concentrated by Ni-NTA affinity chromatography. This step also successfully removed proteolysed by-products (not shown). Starting from 200 mg of denatured protein each, 7.7 mg of mIL-21 and 1.2 mg of hIL-21 were obtained after refolding and affinity chromatography.

The proteins were incubated with TEV protease to remove the N-terminal purification tags and further purified by size exclusion chromatography (Fig. 2). The proteins were eluted as single monodispersed peaks and at an elution volume typical for a 15 kDa globular molecule (Fig. 2A). After this final purification step, 3 mg of purified mIL-21 and 0.25 mg of hIL-21 were obtained. The preparations were >95% pure as determined by silver-staining (Supplementary Fig. 1A).

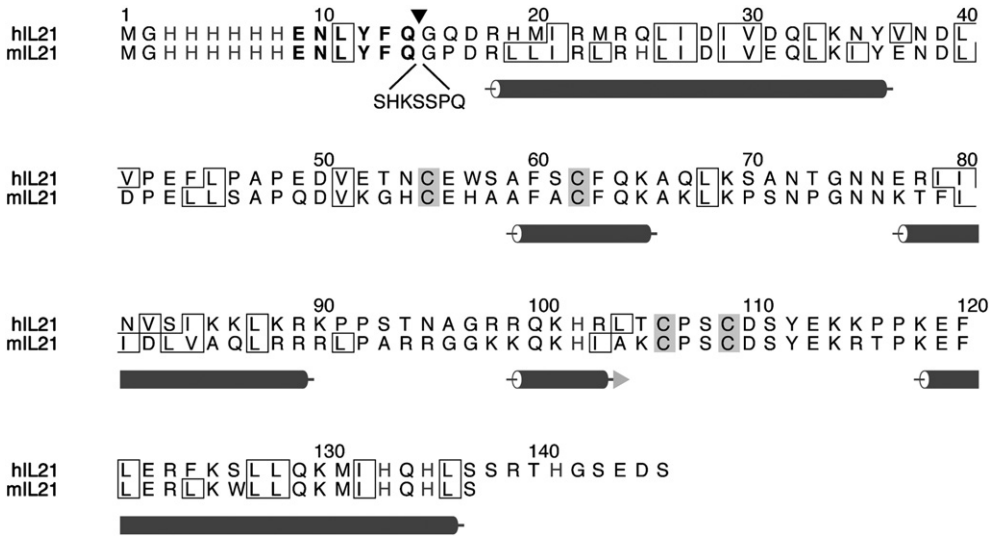

Fig. 1. Sequences of human and murine interleukin-21 expression constructs. The proteins were expressed with N-terminal hexa-histidine and TEV (in bold) tags. TEV protease cleaves the polypeptide chain between residues 14 and 15 (triangle). Secondary structure predictions were generated using Jpred (Cole et al., 2008). Helical (dark tubes) and beta-sheet (light arrows) regions are indicated.

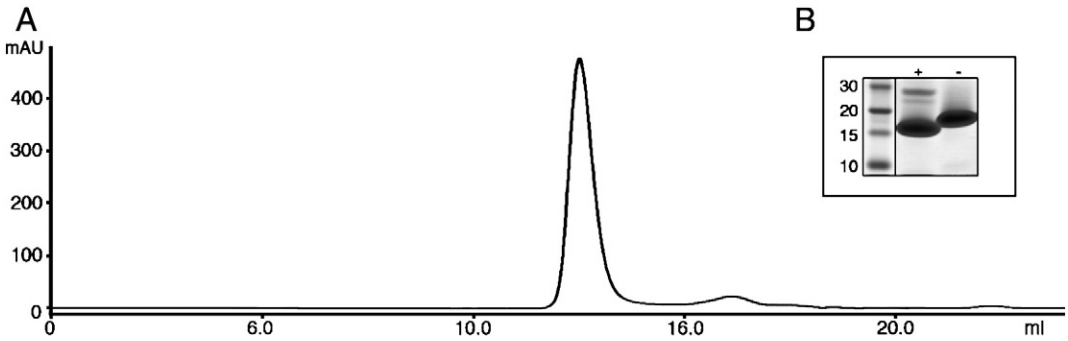

Fig. 2. Purification of interleukin-21 (mIL-21 shown). A) Size exclusion chromatography. The cytokine was purified on a HR75 gel-filtration column (elution was monitored by absorption at 280 nm). B) Protease digest. IL-21 was incubated with (+) or without (−) TEV protease overnight. Digests were analysed by SDS-gel electrophoresis. Faint higher bands correspond to the 27 kDa TEV protease, lower bands to the 14.9 kDa (cleaved) and 16.6 kDa (uncleaved) cytokine.

2.2. IL-21 characterization

To test the bioactivity of the purified IL-21 we used cell proliferation assays, which also allowed us to benchmark our cytokines against IL-21 obtained from a commercial supplier. The assay was based on incubation of two BAF3 bone-marrow-derived cell lines with either hIL-21 or mIL-21 for proliferation. The cell lines had been created by transfection with human/murine interleukin-21 receptor and are dependent on the respective cytokine for proliferation (Yu et al., 2007). This experiment (Fig. 3) revealed that the purified cytokines (black bars) display high bioactivity, comparable to that of commercially available IL-21 (white bars).

Endotoxin levels of the purified cytokines were determined using a standard Limulus Amebocyte Lysate (LAL) assay. The proteins contained low (but detectable) amounts of endotoxin at levels of approximately 0.1 EU/μg, which is below the levels guaranteed by commercial suppliers (usually 1 EU/μg) and suitable for most *in vivo* applications. Endotoxin levels can be further reduced by adding an additional cation-exchange step to the purification protocol (not shown).

2.3. Selection of anti-IL-21 antibody fragments by phage display

The availability of milligram quantities of pure, recombinant IL-21 will allow further characterization of the cytokine and aid in the production of specific affinity reagents. Here we describe the selection of human antibody fragments by phage display (Lee et al., 2007) as an example. For this purpose, human IL-21 was immobilized by adsorption to plastic and a human single-chain Fv (scFv) library (Holt et al., 2000) was selected against the antigen. After three rounds of selection, clones were screened by soluble fragment ELISA (Marks et al., 1991) and 44/60 of them bound to human IL-21. These were sequenced, revealing a set of nine non-redundant antibody fragments. Two of the antibody fragments were further characterized by surface plasmon resonance; they bound to human IL-21 with dissociation constants (K_D) of approximately 0.2 μM and 5 μM. While the higher affinity fragment showed no binding to denatured antigen (not shown), the second antibody fragment specifically detected hIL-21 in Western blot analysis, with no cross-reactivity to mIL-21 observed (Supplementary Fig. 1B).

2.4. Conclusions

The methods described in this publication allow the cloning, expression and purification of murine and human interleukin-21. The cytokines are free of modifications (such as purification tags or N-terminal methionines), pure and highly active as determined by endotoxin and cell proliferation assays. We conclude that the availability of milligram quantities of interleukin-21 will aid in structural, biochemical and physiological analyses of this key immunomodulatory protein.

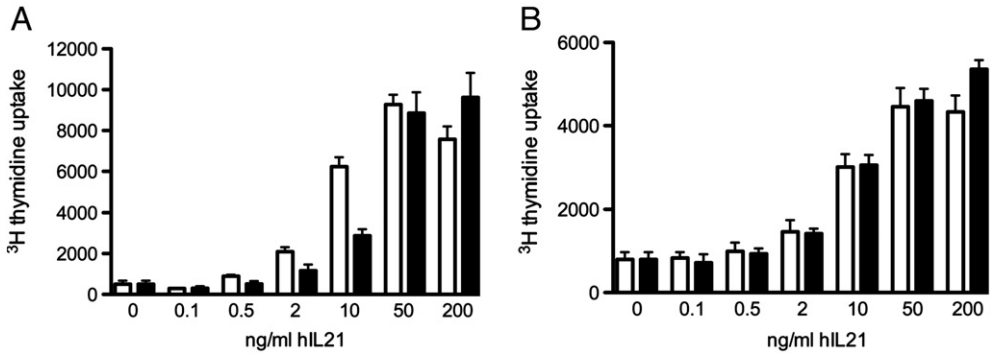

Fig. 3. Interleukin-21 bioactivity. IL-21 was tested using Baf3 cell lines dependent on either human (A) or murine (B) IL-21 (Yu et al., 2007). Cell proliferation was analysed by ^{3}H-thymidine uptake (all samples were tested in triplicate). Shown are purified IL-21 preparations (black bars), as well as controls purchased from a commercial supplier (white bars).

Supplementary materials related to this article can be found online at doi:10.1016/j.jim.2010.08.008.

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