Article

Expression, Purification and Identification of Recombinant Mouse Interleukin 21 Protein in *E. coli*

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Interleukin 21 (IL-21) is a novel type I cytokine that is significantly homologous to IL-2, IL-4 and IL-15. Its receptor complex contains γc chain which is also a component of receptors for IL-2, IL-4, IL-7, IL-9 and IL-15, so there may be overlapping or relevancies in their biological functions. IL-21 is capable of co-stimulating mature T cells, B cells, NK cells, and of stimulating CD16 expression on the surface of NK cells to induce ADCC in innate immune response. It can also strengthen the anti-tumor effect of the cellular immunity, especially *via* enhancing the activities of NK and antigen specific CTL cells. Thus, IL-21 is a potential useful therapeutic molecule for immunotherapy of malignancies, by eliciting innate and adaptive anti-tumor immune responses in tumor-bearing hosts. In order to study the biological functions of IL-21, we constructed a mIL-21 prokaryotic expression plasmid and expressed the recombinant mIL-21 protein in *E. coli* in present study. The recombinant plasmid pET28a/mIL-21 with a carboxyl terminal His-tag was subcloned from the pcDNA3.1/mIL-21 and expressed in *E. coli.* The induced protein was detected by SDS-PAGE, and identified by Western-blot assay with anti-mIL-21 antibody. The recombinant protein was purified *via* Ni^+ affinity chromatography, and renatured with GSH/GSSG system. Our mouse T cell proliferation experiment showed that the recombinant mIL-21 protein could enhance the mouse T cell proliferation either by itself alone or in the presence of Con A. *Cellular & Molecular Immunology*. 2006;3(4):311-315.

Key Words: interleukin 21, gene expression, protein purification

Introduction

Interleukin (IL)-21 is a novel four-helix-bundle type I cytokine which has been recently characterized and described (1). Produced exclusively by activated $CD4^+$ T cells, IL-21 is significantly homologous to IL-2, IL-4, and IL-15. Its receptor, IL-21R complex, is composed of a ligand-binding subunit and an indispensable signal transduction subunit - the common γ chain (1-3). IL-21 is capable of co-stimulating mature T cell proliferation with anti-CD3 antibody, co-stimulating B cell activation with anti-CD40 antibody, and activating NK cell lineages prestimulated with IL-15 or fms-like tyrosine kinase 3 ligand (Flt3L) (1). IL-21 also elevates cytotoxic activity and interferon-γ (IFN-γ) production of NK cells (4, 5). More recently, Chen et al. have shown that IL-21 gene therapy could dramatically enhance the host cell-mediated immunity, especially NK and CTL activities, thus suppressing the tumor growth in mouse tumor models (6-8), which suggests that, with the ability of eliciting innate and adaptive anti-tumor immune responses in tumor-bearing hosts, IL-21 is a potential useful therapeutic molecule for immuno- therapy of malignancies. In this research, we constructed murine IL-21 (mIL-21) prokaryotic expression plasmid, and applied it to expression, purification and identification of the mIL-21 protein in *E. coli* for further research.

Materials and Methods

Materials

The *pfu* polymerase and restriction endonucleases were purchased from Promega (Madison, WI). DL2000 DNA marker and T4 DNA ligase were obtained from Takara (Otsu,

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Abbreviations: IL, interleukin; IPTG, Isopropyl-β-D-Thiogalactoside; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliun bromide; SDS-PAGE, sodium dodecyl sulfate poly-acry-lamide gel electrophoresis; DMSO, dimethyl sulfoxide; LB, Luria Betani medium.

Shiga, Japan). DNA purification kit and GuNTA affinity chromatography kit were purchased from Novagen (Madison, WI). RPMI 1640 was purchased from Invitrogen (Carlsbad, CA). The mIL-21 Western blotting kit was obtained from Genway (San Diego, CA). All the kits were used as recommended by the supplier's manual. Other chemicals were all of analytical grade.

Mice and cell lines

BALB/c mice of 6-8 weeks old were purchased from the University of Science and Technology of China. The mouse splenocytes were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 100 U/ml penicillin-G and 100 μg/ml streptomycin sulfates at 37°C in 5% CO_2 atmosphere.

Plasmids and primers

Plasmid pET28a was gifted by Dr. Guo Wei in the School of Life Science and Technology, China Pharmaceutical University. Plasmid pcDNA3.1/mIL-21 was constructed in our previous studies (6). The mIL-21 specific sense primer 5'-TGT CGC TAG CTC CTG GAG ACT CAG TTC TG-3' and anti-sense primer 5'-CCG GAA GCT TCT AGG AGA GAT GCT GAT G-3' were designed according to the published sequences and synthesized by Invitrogen Company (1). The underline sequences in the sense and anti-sense primes were cut sites of *Nhe* I and *Hind* III respectively, which were corresponding sites in the multiple cloning sites of pET28a. The PCR product was around 460 bp.

Generation of recombinant pET28a/mIL-21 constructs and sequencing

In our previous reports, the murine IL-21 cDNA was reverse transcripted from extracted total RNA of mouse splenocytes activated with 5 mg/ml Con A for 48 h, and inserted into the recombinant plasmid pcDNA3.1/mIL-21. The new PCR products encoding mIL-21 were amplified in the system containing the *pfu* polymerase and pcDNA3.1/mIL-21 as the template. The purified products cut by *Nhe* I and *Hind* III were inserted into the pET28a vector to construct the recombinant plasmid pET28a/mIL-21. The recombinant plasmid was identified with the endonucleases cutting and sequencing (Invitrogen, Shanghai).

Expression and Western blotting analysis of mouse interleukin 21

To express the mIL-21, the pET28a/mIL-21 was transformed into the BL21 (DE3) strain of *E. coli*. The single colony of transformed *E. coli* was picked out and inoculated in a flask containing 500 ml LB medium supplemented with kanamycin (50 μg/ml) and cultured at 37°C until cell density OD600 reached 0.6-0.7. The culture was induced by addition of the final concentration of 0.6 mM IPTG for 3 h at 37 °C. The protein expression was investigated through the 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Western blotting assay was performed with the anti-mIL-21 antibody (Genway Company, USA) after the SDS-PAGE. All steps were performed according to manufacturer's instructions.

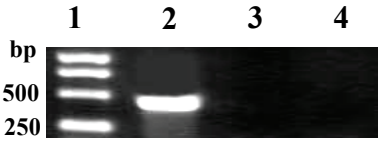

Figure 1. PCR amplification of mIL-21 gene. The PCR products encoding mIL-21 were amplified in the system containing the *pfu* polymerase and different template. Lane 1, DNA marker; Lane 2, PCR products with pcDNA3.1/mIL-21 template; Lane 3, PCR products with pcDNA3.1 template; Lane 4, negative control.

Purification and refolding of mouse interleukin 21

For purification, bacterial pellets were collected by centrifugation at 7,000 g for 10 min, suspended in lysozyme solution (1 mg/ml), harvested by centrifugation again, and suspended with 10 ml of suspending buffer. The cells were disrupted by sonication (5 times for 30 seconds, 30% power). After centrifugation at 12,000 g for 30 min, the supernatant was purified with Ni-NTA slurry according to manufacturer's instructions. The purified protein was dialysis in the refolding buffer containing 5 mM glutathione, 0.5 mM oxidized glutathione and 0.4 M L-arginine. The final products were filtrated with 0.22 μm filter membrane and stored at 4°C.

Identification of mIL-21 bioactivities

T and B cells were isolated from mouse splenocytes as previously described (6). T cell proliferation assay was performed in 96-well plastic culture plate. About 1×10^6 T cells and recombinant mIL-21 (rmIL-21) of various concentrations (from 7 ng to 0.5 μg) were seeded into each well and the total volume was 200 μl. The well without the rmIL-21 was used as negative control, and the well containing 10 mg/L Con A was used as positive proliferation control and coordinated factor control. After incubated at 37°C in 5% CO_2 for 44 hours, the supernatant was discarded and 200 μl MTT was added into each well, then the plate was incubated for another 4 hours. Finally, the plate was measured with the plate reader (Bio-Rad) at OD560 after the supernatant was discarded and 20 μl DMSO was added and shaken for a while.

Statistical analysis

The data were performed using the Student's *t* test for the difference between the experiment group and control group, and $p < 0.05$ was taken as statistically significant.

Results

Amplification of mIL-21 gene and construction of recombinant plasmid

The agarose gel electrophoresis (AGE, 15 g/L) showed the size of the PCR products in the assay of the amplification of

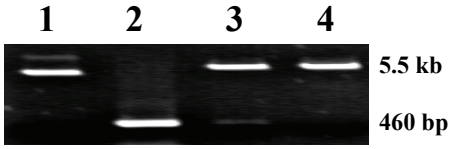

Figure 2. Electrophoresis of pET28a/mIL-21 digested by *Nhe* I and *Hind* III. The recombinant plasmid pET28a/mIL-21 was identified with the endonucleases *Nhe* I and *Hind* III digesting. Lane 1, pET28a/mIL-21; Lane 2, 460 bp DNA marker (PCR product); Lane 3, pET28a/mIL-21 digested by *Nhe* I and *Hind* III; Lane 4, pET28a digested by *Nhe* I and *Hind* III.

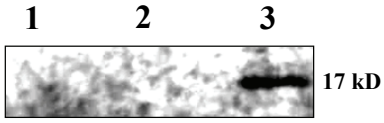

Figure 4. Western bloting detection of IPTG-induced expression of pET28a/mIL-21 in BL21 (DE3). After 12% SDS-PAGE, the bands of each group were transferred to PVDF membrane, then incubated membrane with anti-mIL-21 antibody and the second antibody sequentially, and took the colorimetric assay after adding the DAB and H_2O_2. Lane 1, BL21 (DE3) control; Lane 2, pET28a transformed into BL21 (DE3); Lane 3, pET28a/mIL-21 transformed into BL21 (DE3).

mIL-21 gene was about 460 bp long, which was definitely the same size as predicted (Figure 1). There were two bands in AGE after the recombinant plasmid pET28a/mIL-21 digested by *Nhe* I and *Hind* III, one was the 460 bp inserted sequence, and the other was the vector fragment (5,500 bp). The successful construction of the pET28a/mIL-21 plasmid was identified from the sequencing outcome and the AGE results that these two fragments corresponded respectively to the PCR products and the empty vector (Figure 2).

IPTG induced expression of recombinant protein

After the identified recombinant plasmid pET28a/mIL-21 had been transformed into BL21 (DE3), 0.6 mmol/L IPTG was used to induce the expression for 3 hours and the SDS-PAGE result showed there was one more additional band in recombinant bacteria group than the control, which was just the same size, about 17 kD, as predicted (Figure 3).

Western blotting detection

The Western blotting assay using mIL-21 monoclonal antibody showed that a hybridization band was found only in recombinant bacteria lane (Figure 4), while no band was detected in control group. It suggested that the expressed product of recombinant bacteria was mIL-21 protein.

Purification, renaturation of protein and the identification of mIL-21 bioactivities

After purified by niccolum iron affinity chromatograph, the product was dialyzed for several times and then identified by SDS-PAGE. The recombinant protein was shown only one single band in the electrophoresis assay (Figure 5). The result of MTT showed that the rmIL-21 could stimulate the mouse lymphocyte proliferation independently ($p < 0.05$) or in cooperation with Con A in the splenocyte proliferation assay (Figure 6), and the effect stepped up as the rmIL-21 concentration increased.

Discussion

IL-21, secreted only by T cells in peripheral blood, is one of the cytokines belonging to the γc family. It has attracted more focus recently because of its receptor complex containing γc chain which is also a component of the receptors of IL-2, IL-4, IL-7, IL-9 and IL-15 (2, 3, 9). Meanwhile, IL-21 is highly isogenous with IL-2, IL-15 and IL-4, so there may be overlapping or relevancies in their biological functions. It has been discovered that IL-21 can promote NK cells to differentiate and express CD16 molecule, so as to enable the ADCC effect in the innate immune response (1). Despite that some experiments revealing that IL-21 can not maintain the existence of NK cells for a long time, IL-21 is an important factor influencing the differentiation and growth of NK cells (4). IL-21 also participates in the activation of B cells in the humoral immunity, and can suppress IL-4 mediated antigenic specific generation of IgE (10, 11). Thus the absence of

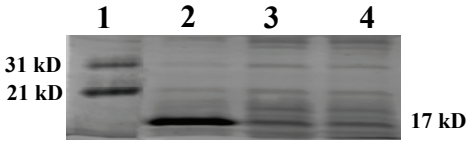

Figure 3. SDS-PAGE detection of IPTG-induced expression of pET28a/mIL-21 in BL21 (DE3). The pET28a/mIL-21 was transformed into the BL21 (DE3) strain of *E. coli*, and induced by 0.6 mM IPTG for 3 h at 37°C. The protein expression was investigated through the 12% SDS-PAGE. Lane 1, protein marker; Lane 2, pET28a/mIL-21 transformed into BL21 (DE3); Lane 3, pET28a transformed into BL21 (DE3); Lane 4, BL21 (DE3) control.

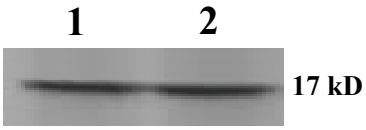

Figure 5. SDS-PAGE detection of rmIL-21 after purification and renaturation. The supernatant of recombinant bacterial cell lysates was purified with Ni-NTA slurry after the sonication. The purified protein was dialysis in the refolding buffer containing 5 mM glutathione, 0.5 mM oxidized glutathione and 0.4 M L-arginine. Both the sample after the purification and renaturation were detected by 12% SDS-PAGE. Lane 1, rmIL-21 product after purification; Lane 2, rmIL-21 product after renaturation.

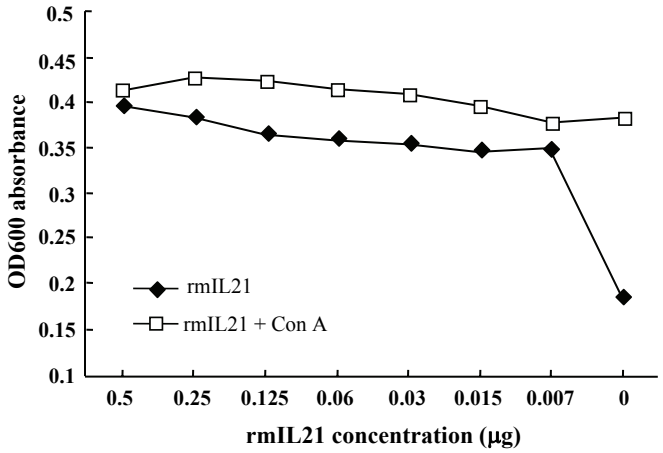

Figure 6. rmIL-21 promotes murine lymphocyte proliferation. About 1×10^6 splenocytes and rmIL-21 of various concentrations (from 7 ng to 0.5 μg) were seeded into each well of plastic culture plate. The wells contained 10 mg/L Con A and rmIL-21 were used as coordinated factor control. The well containing only 10 mg/L Con A was as the Con A positive proliferation control, and the well containing no Con A or rmIL-21 was used as the negative control. Statistical analysis of the data was performed between the experiment group and the negative control.

IL-21 receptors will lead to severe deficiency of IgG secretion. In terms of cellular immunity, IL-21 can enhance the cytotoxic activity of antigenic specific T cells, and strengthen the anti-tumor effect of T cells activated by anti-CD3 antibodies (1, 5). IL-21 secreted by Th2 cells can suppress Th1 cells synthesizing IFN-γ, thus adjusting the balance between Th1 and Th2 (12). IL-21, in cooperation with IL-4, plays the important modulating role of antibody synthesis (11); cooperates with IL-15 to promote the differentiation and growth of NK cells, and to promote the expression of some cytokines in the Th1 cellular immunity (13). Because of the extensive biological activities, IL-21 is expected to exert a substantial contribution in the therapy of immunologic deficiency disease, IgE dependent allergic disorders and cancer, etc.

Chen et al. have used Con A to stimulate murine periphery T cell and amplified mIL-21 cDNA successfully with reverse transcriptase AMV, *pfu* DNA polymerase and primer designed by themselves (6). Moreover, they found that mIL-21 gene therapy could inhibit the growth of murine metastatic lymphoma (7, 8), and the cellular immunity activity of tumor-bearing mice, in terms of the activities of CTL, NK cell and IFN-γ secreting, has been enhanced dramatically. Its mechanism of anti-tumor may associate with the enhancement of the cellular immunity, especially the activities of NK and CTL cells (8). In order to further study the biological functions of IL-21, we planned to construct mIL-21 prokaryotic expression plasmid and expressed it in *E. coli.* The pET prokaryotic expression plasmid is one of the most effective expression systems, taking the advantage of high performance and specific interaction between bacteriophage T7 promoter and T7 RNA polymerase to increase the expression efficacy of exogenous gene in bacteria. The results of this study demonstrated that the recombinant plasmid we constructed can express mIL-21 effectively in *E. coli.* The expressed protein can be purified by niccolum iron affinity chromatograph conveniently because of the six histidines tag. Because mIL-21 protein contains two pairs of cysteines, we take the dialysis system with the renaturation buffer (deoxidize/oxidize glutathione) to recover its natural conformation. The experiments showed that the purified and renatured mIl-21 can promote the proliferation of mouse lymphocytes either by itself alone or in the presence of Con A stimulation, which is consistent with other reference (1). This experiment laid a stable foundation for further IL-21 related research.

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