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Amelioration of DSS-Induced Acute Colitis in Mice by Recombinant Monomeric Human Interleukin-22

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ABSTRACT

IL-22, a pleiotropic cytokine, is known to have a profound effect on the regeneration of damaged intestinal barriers. The tissue-protective properties of IL-22 are expected to be potentially exploited in the attenuation and treatment of colitis. However, because of the disease-promoting role of IL-22 in chronic inflammation, a comprehensive evaluation is required to translate IL-22 into the clinical domain. Here, we present the effective production of soluble human IL-22 in bacteria to prove whether recombinant IL-22 has the ability to ameliorate colitis and inflammation. IL-22 was expressed in the form of a biologically active monomer and non-functional oligomers. Monomeric IL-22 (mIL-22) was highly purified through a series of 3 separate chromatographic methods and an enzymatic reaction. We reveal that the resulting mIL-22 is correctly folded and is able to phosphorylate STAT3 in HT-29 cells. Subsequently, we demonstrate that mIL-22 enables the attenuation of dextran sodium sulfate-induced acute colitis in mice, as well as the suppression of pro-inflammatory cytokine production. Collectively, our results suggest that the recombinant mIL-22 is suitable to study the biological roles of endogenous IL-22 in immune responses and can be developed as a biological agent associated with inflammatory disorders.

Keywords: Interleukin-22; Colitis; Inflammation; Recombinant proteins; Monomer

INTRODUCTION

IL-22, a member of the IL-10 cytokine family, plays a significant role in host defense against microbial infections and is closely associated with a broad range of immunological disorders including psoriasis, Crohn's disease, hepatitis, and rheumatoid arthritis (1,2). IL-22 is characterized by a 6 alpha helix bundle with 2 disulfide bonds (3,4) and is mainly produced in various immune cells such as T helper cells (TH1, TH17, and TH22), type 3 innate lymphoid cells, natural killer T cells, and γδ T cells (5,6). Interestingly, contrary to the original meaning of IL, IL-22 can mediate cellular communication between immune cells and some non-immune cells to induce the expression of tissue-specific effector molecules for cell differentiation and tissue regeneration (5). The biological functions of IL-22 are triggered by the formation of a binary complex between IL-22 and IL-22 receptor 1 (IL-22R1), and the

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Conflict of Interest

The authors declare no potential conflicts of interest.

Abbreviations

CD, circular dichroism; DLS, dynamic light scattering; DSS, dextran sodium sulfate; EK, enterokinase; hrIL-22, human recombinant IL-22; IBD, inflammatory bowel disease; IL-22R1, IL-22 receptor 1; IL-10R2, IL-10 receptor 2; IPTG, isopropyl-β-D-thiogalactopyranoside; ISC, intestinal stem cell; mIL-22, monomeric IL-22; Ni-NTA, nickel-nitrilotriacetic acid; pSTAT3, phosphorylated-signal transducer and activator of transcription 3; rIL-22, recombinant IL-22; SEC, size-exclusion chromatography; Trx, thioredoxin; Trx-IL-22, Trx fused human IL-22; TBS, Tris-buffered saline; TBST, TBS containing 0.1% Tween-20; WB, Western blot.

Author Contributions

Conceptualization: Lee JJ, Ko HJ; Data curation: Hong EH, Ryu Y; Funding acquisition: Hong EH, Ryu Y, Lee JJ, Ko HJ; Investigation: Kim S, Hong EH, Lee CK; Methodology: Ryu Y, Jeong H, Heo S; Supervision: Lee JJ, Ko HJ; Writing - original draft: Kim S, Hong HE, Lee CK, Ryu Y; Writing - review & editing: Lee JJ, Ko HJ.

dimer subsequently interacts with IL-10 receptor 2 (IL-10R2) (4,7). The resulting complexes can activate downstream signaling cascades by phosphorylation of JAK1 and STAT3, giving rise to the upregulation of many relevant genes (8,9). IL-22R1 has a high-binding affinity toward IL-22 and is restrictedly expressed on epithelial cells unlike IL-10R2, which is expressed ubiquitously (10,11). Therefore, the major target organs of IL-22 are generally considered as liver, pancreas, lung, colon, intestine, and kidney, in which epithelial cells cooperatively form a continuous physical barrier to allow tightly regulated cellular transport and prevent pathogen infections (5).

As pleiotropic functions of IL-22 have been elucidated, many studies have demonstrated the therapeutic potential of IL-22 in liver and pancreas regeneration, infection prevention, and graft-versus-host disease (12,13). Recently, a recombinant human IL-22-Fc dimer (F-562) was evaluated in phase II clinical trials to provide evidence of whether IL-22 could be a promising agent to treat patients with alcoholic hepatitis (14,15). This study clearly demonstrated that the engineered IL-22 resulted in improved liver injury-related scores in patients by showing both decreased plasma inflammatory markers and elevated regenerative markers (14). More importantly, IL-22 is estimated to be promising for treatment of inflammatory bowel disease (IBD) such as Crohn's disease and ulcerative colitis (16,17). Several studies provided evidence for the protective roles of IL-22 in IBD by promoting regeneration of intestinal stem cells (ISCs) and attenuating dysregulated immune responses (18,19). As mentioned above, IL-22 can selectively activate the STAT3 pathway, thereby upregulating the expression of several target genes primarily involved in cell survival and proliferation. Consequently, in damaged intestinal tissue, IL-22 substantially promotes the re-establishment of the intestinal epithelial barrier integrity and function (20,21). Based on the molecular mechanism, it was suggested that IL-22 is essential for epithelial maintenance and regeneration in intestinal injury (22,23). As the beneficial effects of IL-22 have been revealed, IL-22-mediated STAT3 activation is attracting attention as a promising therapeutic option for intestinal wound healing and treatment of inflammatory disorders such as necrotizing enterocolitis (13,24,25).

Despite the clinical significance of IL-22, the effective production of human recombinant IL-22 (hrIL-22) in bacteria has not yet been established. Therefore, recombinant IL-22 (rIL-22) has been mainly obtained by a refolding method from bacterial inclusion bodies due to its extremely low solubility (26,27). Moreover, because the renaturation process is a complex and labor-intensive procedure, it is difficult to optimize and perform at a laboratory scale for research purposes (28). In this study, we present a cost-effective production of soluble hrIL-22 in bacteria without a solubilization step and evaluate its biological activities through *in vitro* cell-based assay and *in vivo* mouse studies. To summarize briefly, to promote the correct formation of 2 disulfide bonds of IL-22, we genetically inserted a thioredoxin (Trx) tag at the N-terminus of IL-22, and the resulting IL-22 exhibited highly enhanced soluble expression. Through a series of downstream processing, monomeric IL-22 (mIL-22) was finally purified with very high purity (up to 95%). We subsequently characterized the structural and biological properties of mIL-22 by several biochemical studies, including circular dichroism (CD) analysis and cell proliferation assay. Finally, the therapeutic potential of rIL-22 was successfully demonstrated in a dextran sodium sulfate (DSS)-induced acute colitis mouse model. Details are reported herein.

MATERIALS AND METHODS

Molecular cloning

Codon optimized human IL-22 gene for *Escherichia coli* was synthesized (Integrated DNA Technologies IDT, Coralville, IA, USA) and amplified through PCR using the following primers (restriction enzyme sites are underlined): 1) forward 5′-ATATATCCATGGCTCCCATTTCGAGTCATTGTCG-3′ 2) reverse 5′-ATATATCTCGAGGATACAAGCGTTGCGTAAGG-3′. Forward and reverse primers bear *NcoI* and *XhoI* cleavage sites, respectively. For tagging Trx at the N-terminal end of IL-22, the amplified IL-22 gene was cloned into a pET32a (+) vector (Novagen, Madison, WI, USA) using 2 restriction sites as shown in **Fig. 1B**. The final construct of rIL-22 possessed both a Trx tag and a 6× histidine tag, allowing for increased soluble expression and facilitated affinity purification.

Protein expression and purification

The expression plasmid vector pET32a (+) containing Trx fused human IL-22 (Trx-IL-22) gene was transformed into Origami B (DE3) competent cells, then the transformed cells were incubated on LB broth agar plates containing ampicillin (100 μg/ml), kanamycin (50 μg/ml), and tetracycline (10 μg/ml) at 37°C overnight. A single colony was picked, inoculated into 10 ml of LB broth medium, and pre-cultured at 37°C overnight. Pre-cultured cells were diluted 100-fold in 1,000 ml of LB medium with all 3 types of antibiotics. When the OD_{600} of the cultured cells reached 0.5–0.7, isopropyl-β-D-thiogalactopyranoside (IPTG) was added at final concentration of 0.5 mM to induce protein expression. The ideal induction time in this study was approximately 21 h at 18°C. Cultured cells were harvested by centrifugation at 6,000 rpm for 10 min at 4°C. The pelleted cells of 1,000 ml culture were re-suspended with 40 ml of lysis buffer (50 mM NaH_2PO_4 [pH 8.0], 300 mM NaCl, and 10 mM imidazole).

Prior to cell disruption, protease inhibitor cocktail (Roche, Pleasanton, CA, USA) was added, and sonication was conducted at 40% amplitude for 1 h. After sonication, cell lysate was separated by centrifugation at 13,000 rpm for 1 h at 4°C. Inclusion bodies were solubilized in 8 M urea for SDS-PAGE analysis. The soluble fraction was filtered with a syringe filter with 0.22 μm pore size, and further purified by affinity chromatography. First, nickel-nitrilotriacetic acid (Ni-NTA) agarose resin (Qiagen, Hilden, Germany) was equilibrated in a disposable 5 ml polypropylene column (Thermo Fisher Scientific, Waltham, MA, USA) with lysis buffer for 10 column volume. The filtered solution was applied to the buffer-equilibrated Ni-NTA column. The column was washed 3 times with 5 column volume of a wash buffer (50 mM NaH_2PO_4 [pH 8.0], 300 mM NaCl, and 20 mM imidazole). Bound proteins were eluted by elution buffer (50 mM NaH_2PO_4 [pH 8.0], 300 mM NaCl, and 250 mM imidazole). Subsequently, size-exclusion chromatography (SEC) using HiLoad® 16/600 Superdex® 75 column (Cytiva, Marlborough, MA, USA) was performed to increase purity of Trx-IL-22. Overall purity and concentration of the purified protein were determined by SDS-PAGE and Bradford assay, respectively.

Trx tag removal

To remove Trx tag from IL-22 protein, enterokinase (EK) was employed to cleave its recognition site located between Trx and IL-22 (29). Buffer exchange of purified Trx-IL-22 was performed with an EK reaction buffer (20 mM Tris-HCl [pH 8.0], 50 mM NaCl and 2 mM $CaCl_2$) using a PD-10 desalting column (GE Healthcare, Chicago, IL, USA). Light chain of human EK (10 μg/ml) was incubated with Trx-IL-22 (0.4 mg/ml) for 2 h at 25°C. Following enzymatic reaction, protease inhibitor cocktail (Roche) was treated to the Trx-IL-22 solution

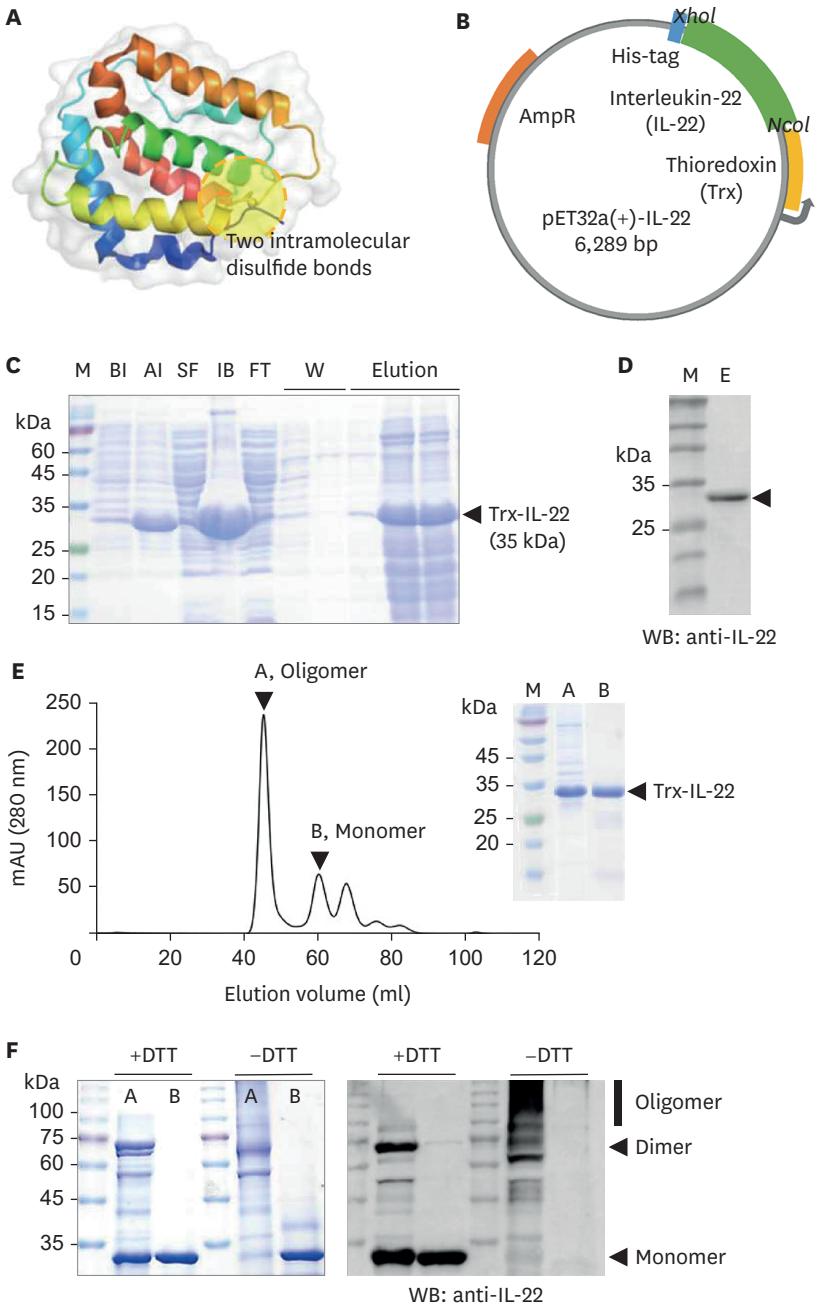

Figure 1. Bacterial expression and purification of Trx-tagged human IL-22. (A) The ribbon representation of the folded structure of IL-22. Two intramolecular disulfide bonds were indicated within yellow transparent circle. (B) A schematic diagram of a designed bacterial expression vector pET32a (+) for the production of IL-22 with Trx. (C) SDS-PAGE analysis of Trx-IL-22 protein which purified by using Ni-NTA resin. (D) WB analysis of the purified Trx-IL-22 in elution fraction shown in Fig. 1C using anti-IL-22 primary Ab. (E) SEC profile of Trx-IL-22 protein. Peak A and B containing Trx-IL-22 oligomer and monomer, respectively, were identified by SDS-PAGE analysis. (F) WB analysis of IL-22 eluted at peaks A and B of SEC in both absence and presence of a reducing agent, 100 mM DTT. No mIL-22 band was detected under non-reducing conditions (lane B), possibly because epitopes of anti-IL-22 Ab were masked through conformational folding formed by correct disulfide bonds.
BI, before isopropyl-β-D-thiogalactopyranoside induction; AI, after isopropyl-β-D-thiogalactopyranoside induction; SF, soluble fraction; IB, inclusion body; FT, flow through; W, washing fraction.

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to prevent unwanted non-specific protein cleavage and degradation. Buffer exchange was conducted with A buffer (20 mM Tris, pH 7.8), and anion exchange chromatography was sequentially carried out to isolate IL-22 from the EK treated solutions. IL-22 and other proteins were separated through a HiTrap Q FF column (Cytiva) under a linear salt gradient condition from 0% to 50% B buffer (20 mM Tris, 1 M NaCl, and pH 7.8). Respective eluted peaks were analyzed by SDS-PAGE, and all fractions containing IL-22 were concentrated using a 10 kDa centrifugal filter (Merck Millipore, Burlington, MA, USA).

In vivo mouse models

C57BL/6 background female 8-week-old mice were purchased from Koatech (Pyeongtaek, Korea). All experiments were performed by the Institutional Animal Care and Use committee of Kangwon National University (IACUC admission number KW-200831-1) and the mice were bred during experiment in the Animal Laboratory Center of Kangwon National University. For acute colitis induction, mice were treated with 2.5% dextran sulfate sodium (wt/vol, molecular weight 36–50 kDa; MP Biomedicals, Solon, OH, USA) in drinking water for 5 consecutive days with 4 additional days of tap water only. We assessed the induction of colitis through body weight changes. Colon length was assessed by millimeter ruler on the 9 days of the experiment.

ELISA

Cytokines and chemokines including mouse IL-1β, IL-6, IL-17A, CXCL1, and CCL2 were measured using Mouse Uncoated ELISA Kit (Invitrogen, Vienna, Austria) and mouse CXCL1/KC DuoSet ELISA (R&D systems, Minneapolis, MN, USA). Briefly, mouse colon tissues were homogenized using Minilys personal homogenizer (Bertin, Montigny-le-Bretonneux, France). Tissue homogenate was centrifuged and used only supernatant. Immuno-plates (Thermo Fisher Scientific) were coated with capture Ab at 4°C for overnight and Ab-coated wells were blocked with 1× assay diluent for 1 h at 20°C. Standard and sample were diluted and put into each well at 4°C for 18 h. The detection Abs conjugated with HRP were applied into wells and wells were developed with 1× TMB substrate. The absorbance was measured at 450 nm by using SpectraMax i3 (Molecular Devices, San Jose, CA, USA).

CD

CD spectrum of IL-22 was measured at 25°C with J-1500 CD spectropolarimeter (Jasco, Tokyo, Japan) using a 1 mm path cuvette. IL-22 (0.5 mg/ml) was dissolved in a tris-based buffer (20 mM Tris [pH 7.8] and 75 mM NaCl). The collected CD data were analyzed by a K2D3 server (http://cbdm-01.zdv.uni-mainz.de/~andrade/k2d3) to estimate the secondary structure of IL-22.

Dynamic light scattering (DLS)

The size of oligomeric and monomeric Trx-IL-22 proteins were measured by Zeta-potential Particle Size Analyzer (Zetasizer Nano ZSP; Malvern Panalytical, Malvern, UK) with 12 mm Square Polystyrene Cuvettes (DTS0012; Malvern Panalytical). Each sample at 0.2 mg/ml was filtered prior to evaluation.

Western blot (WB)

Purification of rIL-22 was demonstrated through WB analysis. Trx-IL-22 and tag-removed IL-22 were loaded onto 15% SDS-PAGE, transferred to a polyvinylidene fluoride membrane. After masking the membrane with a blocking buffer (Tris-buffered saline [TBS] containing 0.1% Tween-20 and 2% BSA) for 30 min, the membrane was incubated with mouse anti-human IL-22 Ab (1 μg/ml; R&D systems) overnight at 4°C. The membrane was washed

3-times with TBS containing 0.1% Tween-20 (TBST) and incubated with HRP-conjugated mouse IgG kappa binding protein (1:5,000; Santa Cruz Biotechnology, Dallas, TX, USA) for 2 h at room temperature. After washing the membranes with TBST, protein bands were detected with a chemiluminescent substrate (BIOMAX, Derwood, MD, USA) through a CCD Imager (GE Life Sciences, Marlborough, MA, USA).

To verify IL-22 induced STAT3 phosphorylation, human colon cancer HT-29 cells (ATCC, Manassas, VA, USA) in RPMI 1640 media (Welgene, Gyeongsan, Korea) were seeded at 1×10^6 cells/well on a 6-well plate. After 24 h, 10, 100 and 1,000 ng/ml of IL-22 diluted in serum-free media were treated to HT-29 cells for 15 min and 30 min. Cells were washed twice with PBS and lysed with RIPA buffer (Biosolution, Seoul, Korea) containing protease inhibitor and phosphatase inhibitor (Gendepot, Katy, TX, USA) on ice. Lysed cells were collected using a scrapper and soluble lysate was obtained by centrifugation at 13,000 rpm for 15 min at 4°C. Total protein concentration in each sample was determined by the bicinchoninic acid assay (Thermo Fisher Scientific). Samples (20 μg) were loaded onto 8% and 12% PAGE to detect total STAT3 and phosphorylated-STAT3 (pSTAT3), and β-actin, respectively. Mouse anti-STAT3 (1:1,000; Cell Signaling Technology, Danvers, MA, USA), mouse anti-pSTAT3 (Tyr705, 1:2,000; Cell Signaling Technology), and rabbit anti-β-actin (1:5,000; Proteintech, Rosemont, IL, USA) Abs were employed as a primary Ab. Subsequent reactions followed the same protocols as described above. For the β-actin detection, membrane was blocked with 5% skim milk (BD Difco, Franklin Lakes, NJ, USA) in TBST for 20 min, and then protein band was developed through mouse anti-rabbit IgG-HRP (1:5,000; Santa Cruz Biotechnology).

Cell proliferation assay

HT-29 cells were seeded into a 96-well plate (SPL, Pocheon, Korea) at a density of 1×10^4 cells/well. After incubation for 24 h, different concentrations of IL-22 (from 37.5 ng/ml to 600 ng/ml) were treated to each well for 48 h and 72 h. Cell proliferation was estimated using a WST-8 reagent (Biomax, Seoul, Korea) which was incubated with cells for 30 min to 1 h. The absorbance was measured at 450 nm with a microplate reader (BioTek, Winooski, VT, USA).

Statistical analysis

Data analysis was performed using Graphpad Prism 9 (Graphpad Software Inc., San Diego, CA, USA) software and Flowjo V10. The data were compared using a student's *t*-test between the 2 groups and one-way ANOVA followed by *post hoc* tests (Tukey's test) was used to compare more than 2 groups. Statistical significance is represented by $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

RESULTS

Bacterial production of hrIL-22

IL-22 belongs to the class II cytokine family and is described as a compact 6-helix bundle (**Fig. 1A**) (4). The correct formation of 2 disulfide bonds between amino acids 40–132 and amino acids 89–178 is important to retain the structural integrity and biological activity of IL-22 (30). To promote the formation of native disulfide bridges, Trx was genetically fused with human IL-22, and the resulting gene was inserted into a pET32a bacterial expression vector (**Fig. 1B** and **Supplementary Fig. 1**). We carried out the bacterial expression of the recombinant Trx-IL-22 in the DE3 *E. coli* strain. As shown in **Fig. 1C**, overexpression of Trx-IL-22 in bacteria was clearly observed in the protein band after IPTG induction (AI) with a molecular weight of 35 kDa. Although most of the expressed IL-22 aggregated in the form of

inclusion body (IB) as previously reported, we successfully obtained about 4 mg of soluble Trx-IL-22 per liter culture. The collected elution fraction of Trx-IL-22 was subjected to WB analysis to verify that the purified protein is IL-22 by using an anti-human IL-22 Ab (**Fig. 1D**).

To increase the purity of Trx-IL-22, SEC was subsequently performed (**Fig. 1E** and **Supplementary Fig. 2A**). Unexpectedly, we observed that Trx-IL-22 eluted in 2 main peaks (A and B). Based on a calibration curve of SEC (**Supplementary Fig. 2B**), we identified that peak B with an estimated molecular weight of 42 kDa is a monomeric form of Trx-IL-22 while peak A is most likely soluble oligomeric aggregates (calculated as 106 kDa). To verify the oligomeric state of rIL-22, we determined the molecular size of eluted IL-22 at peak A and B by using DLS (**Supplementary Fig. 2C**). Compared with the monomer size of peak B (diameter 8.7 nm), peak A demonstrated significantly increased size of IL-22 (diameter 26.0 nm to 105.4 nm). The DLS data were in good agreement with SEC, providing that rIL-22 tends to form soluble aggregates of various sizes. Furthermore, we found that in the solution of peak A, insoluble aggregates were gradually generated in a time-dependent manner, but this phenomenon did not occur in peak B (**Supplementary Fig. 3A and B**). After 14 days of incubation, WB analysis clearly showed the soluble form of the multimeric IL-22 was significantly reduced compared to the partially digested monomer, owing to the formation of insoluble aggregates (**Supplementary Fig. 3C**). The results suggest that the oligomeric IL-22 is more unstable than the monomeric form.

Previous studies have shown that IL-22 is generally known to be functional as a monomer, but can assemble into dimeric forms at high protein concentrations through hydrophobic and noncovalent interactions (31,32). However, there is no report of IL-22 forming multimeric soluble aggregates. We thus investigated if the oligomeric nature of recombinant IL-22 is the result of non-specific weak interactions or the result of improper formation of intermolecular disulfide bonds. Consequently, WB analysis was performed under both reducing (100 mM DTT) and non-reducing conditions (**Fig. 1F**). When no DTT was introduced, multimeric bands of IL-22 were observed in lane A (oligomeric IL-22, peak A), whereas a negligible oligomeric band was detected in lane B (monomeric IL-22, peak B). In addition, it was identified that the residual band of the dimer-sized IL-22 (lane A) under the reducing conditions almost disappeared when tris(2-carboxyethyl)phosphine, a more powerful reducing agent than DTT, was additionally treated (**Supplementary Fig. 4A**). Taken together, these data indicate that formation of non-native disulfide bridges between IL-22 plays an important role in promoting oligomerization and aggregation.

Bioactivity comparison of monomeric and oligomeric IL-22 *in vivo*

To compare the activity of oligomeric and mIL-22, we preliminarily evaluated the therapeutic potential of eluted IL-22 in 2 well-separated peaks (A and B) using SEC in a mouse model of DSS-induced acute colitis. Compared to the control group, the mice treated with 2.5% DSS lost 20% of their body weight by day 9 and the colon length was shortened as the result of acute colitis (**Fig. 2A and B**). Interestingly, mice injected with monomeric Trx-IL-22 of peak B showed notable protection from DSS-induced body weight loss and colon length shortening, which was not observed in the multimeric Trx-IL-22-treated group (**Fig. 2B and C**). The result suggests that the purified monomeric Trx-IL-22 has bioactivity to help repair and proliferation of injured colonic epithelial cells, whereas the oligomeric form of Trx-IL-22 was found to have no effect on ameliorating acute colitis in the mouse model. We speculate that the oligomeric form exhibited low stability so that it could not play a protective role like the monomeric Trx-IL-22. Therefore, the monomeric Trx-IL-22 was selected for further downstream processes,

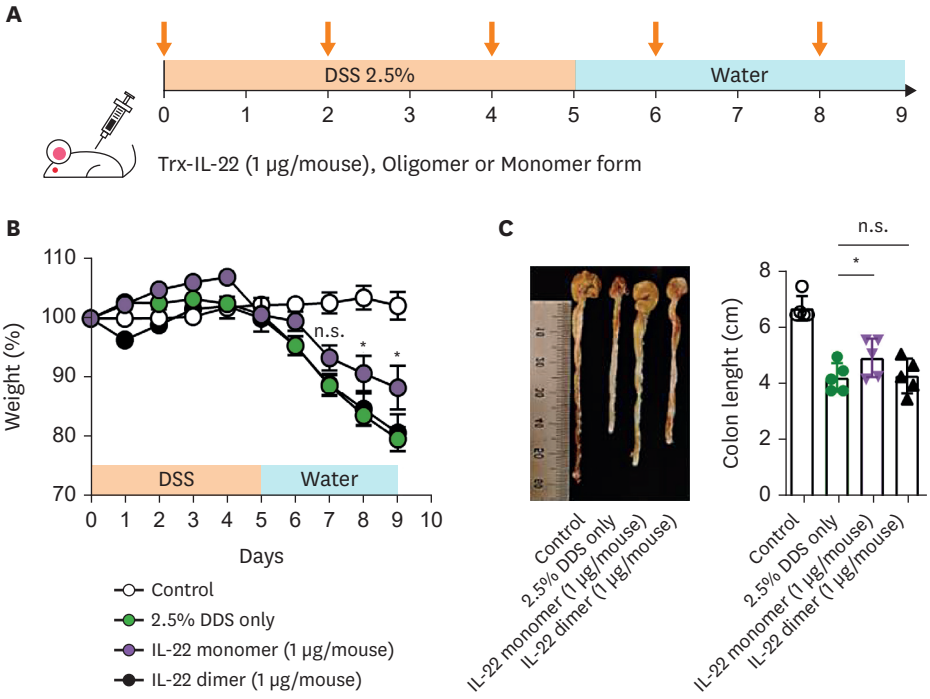

Figure 2. Biological effect by monomeric and oligomeric form of IL-22 in DSS-induced colitis. (A) Experimental scheme of Trx-IL-22 administration during 2.5% DSS-induced colitis progression. (B) The daily percent change of body weight in each group. (C) Representative image and length of the colon. Data are expressed as mean ± SD, n=5 per group (**Supplementary Table 1**).
n.s., not significant.
*p<0.05 (one-way ANOVA followed by Bonferroni test).

including an enzymatic reaction for Trx tag removal and a final polishing step using ion-exchange chromatography to evaluate the therapeutic potential of rIL-22.

Biochemical characterization of monomeric and intact IL-22

For less unwanted effect of recombinant proteins, it is inevitable to remove an unnecessary domain, such as solubilizing tag and affinity tag, from proteins of interest (33,34). The downstream protein purification allows the prevention of unwanted biological reactions and reduces the risk of adverse effects. To eliminate the Trx tag from IL-22, EK was treated with the monomeric Trx-IL-22 in which the Trx tag is flanked by the recognition sequence of EK. First, we determined the optimum incubation time of EK (10 μg/ml) necessary for an effective enzymatic reaction and avoiding non-specific proteolysis. As a result, it was found that an incubation time of 2 h is sufficient to completely remove the Trx tag from IL-22 by SDS-PAGE and WB (**Fig. 3A** and **Supplementary Fig. 5**). After the enzyme reaction, mIL-22 was isolated through anion exchange chromatography with a linear salt-gradient owing to the significant difference in isoelectric point of IL-22 (6.8) and Trx (4.7). As shown in **Fig. 3B**, mIL-22 was eluted in the first peak (A) with a remarkable high-purity (97.2%). To assess the structural integrity of the resulting mIL-22, we performed CD spectroscopy and revealed that the CD spectra of mIL-22 were highly similar to that of the previously reported data showing α-helical structure (**Fig. 3C**) (4,27). Furthermore, computer prediction of protein secondary structure using CD data clearly showed that mIL-22 is mainly composed of alpha helices (90.8%). Based on these results, we demonstrated that human IL-22 can be effectively produced in bacteria as a monomeric and properly folded form.

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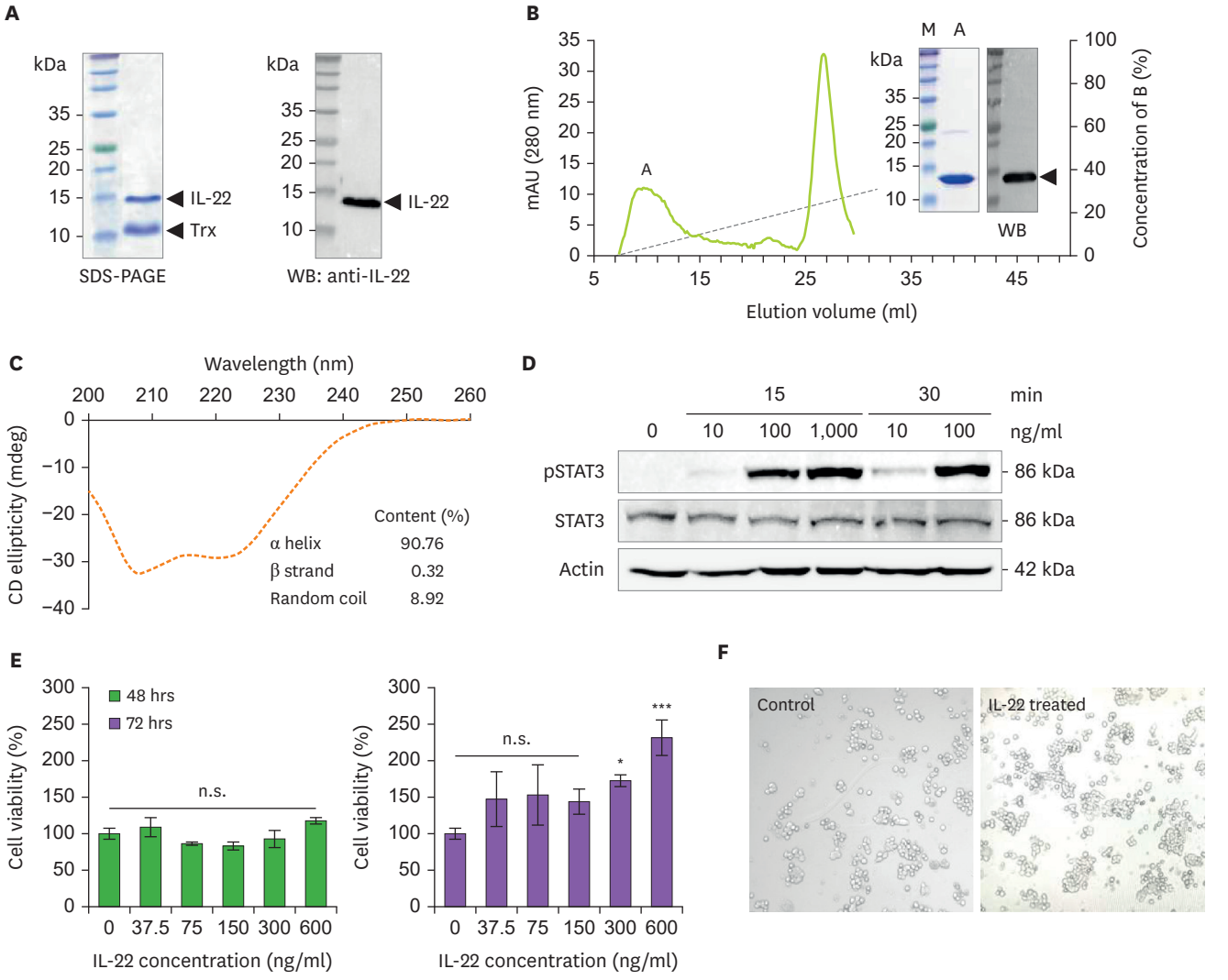

Figure 3. Characterization of mIL-22 and evaluation of its biological activity. (A) SDS-PAGE and WB analysis of Trx-removed mIL-22 protein after EK cleavage reaction. EK recognition sequence is located adjacent to the N-terminus of IL-22. For WB analysis, anti-IL-22 Ab was used as a primary Ab. (B) Anion exchange chromatography profile of purified mIL-22 protein from EK cleavage reaction using a linear NaCl gradient. Peak A containing mIL-22 was verified by SDS-PAGE and WB analysis. (C) CD analysis of mIL-22. The result of secondary structure analysis was shown below the CD spectrum of IL-22. (D) WB analysis of pSTAT3 of HT-29 cells induced by mIL-22 treatment. STAT3, pSTAT3, and actin were detected with increasing stimulation time and concentration of mIL-22. (E) Dose-dependent effects of mIL-22 on proliferation of HT-29 cells. Graph showing the cell proliferation was presented depending on mIL-22 treatment time (48 and 72 h). The data represent the mean ± SD of 3 experiments. (F) Representative micrographs for verifying IL-22 promoted cell proliferation. HT-29 cells were cultured in serum free media with or without mIL-22 for 3 days. Bright-field images were obtained at 10× magnification.
$^{*}p<0.05$, $^{***}p<0.001$ compared with the untreated control (0 ng/ml).

Next, we investigated the biological activity of mIL-22 through cell-based assays. To elicit a biological response, IL-22 preferentially interacts with its cell surface receptors, IL-22R1 and IL-10R2 (7). The formation of oligomeric IL-22/IL-22R1/IL-10R2 complexes triggers the activation of multiple cellular pathways, in particular, STAT3 signaling (10). To confirm the functionality of IL-22, we studied IL-22-mediated STAT3 phosphorylation in HT-29 adenocarcinoma cell line overexpressing IL-22 receptors (IL-22R1 and IL-10R2) (35). WB analysis revealed that IL-22 significantly induced pSTAT3 in time and concentration-dependent manner while there is no detectable band of pSTAT3 before IL-22 treatment (**Fig. 3D**), indicating that recombinant mIL-22 is biologically active. Since STAT3 phosphorylation is closely related to the development and growth of cancer cells, we carried out a cell viability assay to prove whether mIL-22 has the

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ability to stimulate cell proliferation. After 3 days of incubation, we observed that cells treated with mIL-22 at high concentrations (300 ng/ml and 600 ng/ml) exhibited an approximately 2-fold increase in cell viability compared to the untreated group (**Fig. 3E and F**), implying that the functional mIL-22 has the potential as a biological agent for the study of related diseases, tissue regeneration, and wound healing.

Investigation of the ameliorative effect of IL-22 on DSS-induced colitis

To study the biological activity of mIL-22, we first evaluated the effect of mIL-22 alone without DSS treatment (**Supplementary Fig. 6A**). As a result, there is no difference in body weight and colon length in IL-22-treated group as compared with mIL-22-nontreated group (**Supplementary Fig. 6B and C**). The levels of pro-inflammatory cytokine and chemokines including IL-1β, IL-6, IL-17A, CXCL1 and CCL2 were very low and there was no significant difference between IL-22-treated and IL-22-nontreated group (**Supplementary Fig. 6D**).
To induce colitis, mice were administered with 2.5% DSS in the drinking water for 5 consecutive days, and it was replaced with fresh water on day 5 (**Fig. 4A**). DSS treatment significantly decreased body weight (**Fig. 4B**) and colon length of mice (**Fig. 4C**), suggesting severe colitis development in the colon. The levels of pro-inflammatory cytokines and chemokines including IL-1β, IL-6, IL-17A, CXCL1, and CCL2 were also highly elevated by DSS administration (**Fig. 4D**). Next, we treated mice with 1 μg of IL-22 every other day for 5 times starting on the day of DSS administration. The intraperitoneal injection of mIL-22 prevented body weight loss and reduced colon length shortening. In addition, the level of IL-6, IL-17A, and CCL2 was significantly reduced by IL-22 treatment. In summary, we found that systemic injection of IL-22 attenuated DSS-induced colitis in a murine model, suggesting that recombinant mIL-22 could be a potent protein drug candidate to treat colitis.

DISCUSSION

Current treatments for ulcerative colitis include the use of mesalazine, corticosteroids, monoclonal Abs to TNF-α (infliximab, adalimumab), and immunosuppressants (cyclosporine, azathioprine) (36,37). Mesalazine is the first-line treatment for mild ulcerative colitis and is switched to other medications if the disease does not improve (37). However, since ulcerative colitis is difficult to cure, the main goal of colitis treatment is clinical maintenance and symptom alleviation (38,39). In addition, if the condition is severe, a colectomy can be performed, or an immunosuppressant can be used, but both have potential side effects of increased risk of infection or other diseases (40). Therefore, it is necessary to develop a drug that is more effective and has fewer side effects than currently used drugs.

IL-22 is a cytokine with high potential clinical relevance (13) and is secreted from various immune cells, including Th1, Th17, Th22, LTi, and ILC3 (11,13). IL-22 is a major regulatory cytokine for maintaining the homeostasis of epithelial cells in the intestine (41). In this regard, IL-22 regulates intestinal epithelial regeneration and intestinal barrier function by regulating junction protein expression and secretion of antimicrobial peptides and proteins, including S100 and RegIII (13,41,42). Previous studies have shown that IBD patients underwent increases in IL-22 levels (43,44), but there was no dramatic improvement in the disease. This may be due to the insufficient function of IL-22 by the inhibition of IL-22 binding protein (IL-22BP), a natural antagonist of IL-22, and suggests that an extremely large amount of IL-22 may be required to overcome the IL-22BP-mediated inhibition. Indeed, several reports suggested that the administration of IL-22 or IL-22Fc fusion protein exhibited sufficient protection against

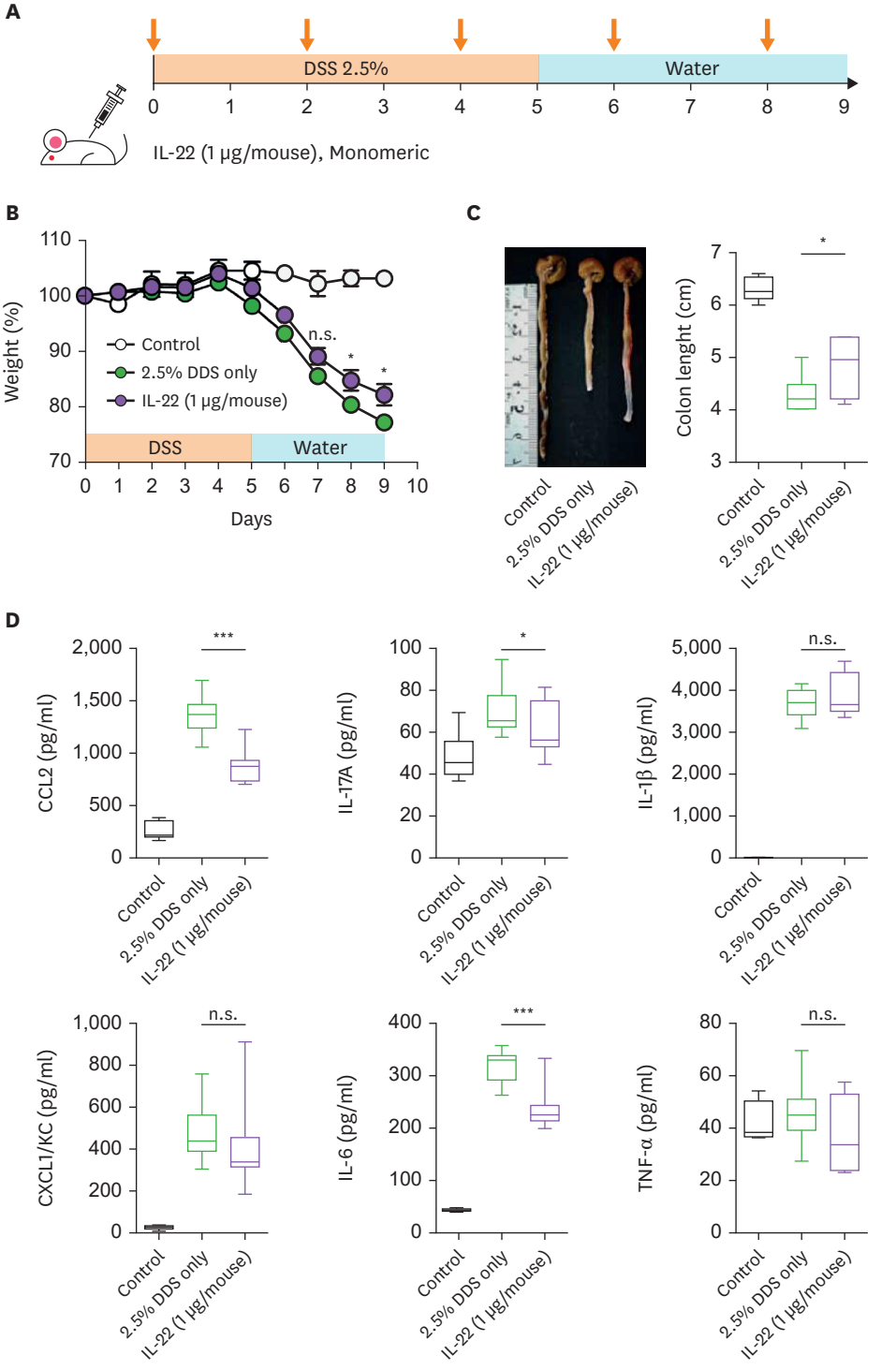

Figure 4. The mIL-22 alleviates against colon damage in DSS-treated mice. (A) The scheme of acute colitis along with mIL-22 administration. (B) The change of body weight percentage. (C) Typical colon image and length. (D) Inflammatory cytokines and chemokines were measured by ELISA in colon. Data are expressed as mean ± SD, n=5 per group.
*p<0.05, ***p<0.001 (one-way ANOVA followed by Bonferroni test).

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DSS-induced colitis in preclinical models (45,46). On the contrary, the blockade of IL-22 by neutralization Ab improved colitis induced by agonist anti-CD40 Ab or DNBS (47), suggesting a complex role of IL-22 in intestinal inflammation and barrier function (41). Recently, an IL-22Fc fusion protein, UTTR1147A, was assessed for its safety in phase I clinical trial (48) and is under evaluation for its efficacy in patients with ulcerative colitis and can potentially provide further information for the clinical applicability of IL-22.

In this study, we presented that monomeric human IL-22 produced in bacteria has therapeutic potential in attenuating interstitial inflammation in mouse models. IL-22, a multifunctional cytokine, is known to have a protective effect in various colitis models (19,49-51). In acute colitis models such as *Citrobacter rodentium*, IL-22 increased the expression of claudin-2, a tight junction protein, which leads to tissue repair and bacteria clearance (52). In addition, IL-22 inhibits prolonged inflammation to reduce damage within epithelial cells and directly targets LGR5+ stem cells to induce regeneration and proliferation of stem cells by STAT3 phosphorylation (18,53,54). Consequently, IL-22 contributes to the protection of intestinal epithelial cells by inducing the regeneration of ISCs in acute tissue injury (18). However, high serum levels of IL-22 can lead to an aggravation of the disease, and the chronic immune disorder in the intestines was regarded to be associated with IL-22-producing cells (55-57). Taken together, the role of IL-22 in the regulation of intestinal inflammation remains elusive, but the current research suggests IL-22 to be beneficial for homeostatic regulation of intestinal immunity and suppresses inflammation.

Several reports suggested that IL-22 promoted tumor growth, especially in established tumors (58,59). This might be associated with the activation of the STAT3 signaling pathway via IL-22-IL-22R interaction. However, IL-22 elicited a protective role in the early stage of tumor initiation (41). Likewise, the administration of anti-IL-22 Ab promoted tumor growth. Thus, although the role of IL-22 in colorectal cancer development is still controversial, the studies imply IL-22 plays a role in tumor initiation.

Given the pleiotropic action of IL-22, understanding the underlying mechanisms of IL-22 in disease is of fundamental importance for maximizing therapeutic benefits and providing insight into potential therapeutic implications. Securing sufficient amounts of bioactive IL-22 protein is a prerequisite for facilitating the evaluation of biochemical and biological properties of IL-22. A previous study presented an expression system for hrIL-22 using *Lactococcus lactis* as a host strain (60). In this system, IL-22 protein was induced by nisin, an antibacterial peptide, and secreted into the culture medium. The secreted IL-22 was shown to have biological activity, but it is very difficult to directly apply non-purified IL-22 into the clinical domain as a therapeutic agent due to uncharacterized impurities. Therefore, for the successful development of cytokine-based biologics, elaborate and well-optimized purification steps that can reduce immunogenicity and unpredictable toxicity are inevitable. To realize the effective production of highly purified IL-22 in bacteria, we focused on establishing a series of purification processes and a bacterial expression system. Moreover, we genetically engineered human IL-22 through codon optimization and Trx fusion to improve the translation efficiency and accelerate disulfide bond formation, allowing high-level soluble expression of bioactive IL-22 in bacteria.

Interestingly, the expressed IL-22 exhibited the form of a biologically active monomer and non-functional aggregates. We found clearly that mIL-22 possesses the ability to attenuate DSS-induced acute colitis, whereas oligomeric IL-22 is prone to aggregation without

observable biological activity *in vivo*. However, the therapeutic efficacy of mIL-22 in colitis is insufficient compared to other chemical reagents, so genetic engineering of IL-22 is required to generate more potent variants. As mentioned above, IL-22 can tightly interact with 2 endogenous proteins, IL-22R1 and IL-22BP in a competitive manner. IL-22/STAT3 signaling is triggered by the complex formation between IL-22 and IL-22R1, but conversely, the signaling pathway is also strongly blocked by IL-22BP, a soluble decoy receptor (61). Accordingly, modulating the binding affinity of IL-22 to IL-22R1 to maximize the agonistic effect of IL-22 can be considered a promising strategy. To this end, we plan to conduct further studies to enhance the binding affinity of IL-22 to IL-22R1 and to modify critical residues of IL-22 in IL-22BP interactions through directed evolution and computational protein design.

In conclusion, we demonstrated that mIL-22 can be effectively produced using a bacterial expression system and ameliorate DSS-induced colitis in mouse models by inhibiting the expression of pro-inflammatory cytokines. Considering the increasing number of patients with inflammatory disorders of the gastrointestinal tract, it is anticipated that the recombinant mIL-22 can be developed as a biological agent with promising therapeutic potential in colitis and other related diseases.

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SUPPLEMENTARY MATERIALS

Supplementary Table 1

Individually measured colon length

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Supplementary Figure 1

Complete amino acid sequences of Trx-IL-22 constructed in this study. IL-22, thioredoxin and histidine tag are colored in green, blue and yellow, respectively. EK recognition sequence is highlighted in red color.

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Supplementary Figure 2

Purification of Trx-IL-22 by size exclusion chromatography. (A) All elution fractions of peak A and B containing Trx-IL-22 shown in **Fig. 1E** were analyzed by SDS-PAGE. (B) Calibration curve of HiLoad 16/60 Superdex 75 column by standard molecular weight proteins (conalbumin: 75 kDa, ovalbumin: 43 kDa, carbonic anhydrase: 29 kDa, ribonuclease A: 13.7 kDa, aprotinin: 6.5 kDa). The calibration curve was plotted using proportion of pores available to the molecule (K_{av}) versus logarithm of the molecular weight (Log Mw). The K_{av} was calculated using the following formula: $K_{av} = (V_e - V_o)/(V_c - V_o)$ where V_e = elution volume,

V_o = column void volume, V_c = geometric column volume. (C) Each eluted sample (peak A and B) was subjected to DLS analysis to estimate the molecular diameter. The DLS data provided that oligomeric IL-22 of peak A has a much larger molecular size than monomeric IL-22 of peak B, suggesting that the oligomeric IL-22 (peak A) can be considered to form soluble aggregates.

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Supplementary Figure 3

Comparison of storage stability of oligomeric and monomeric IL-22. (A) Each purified IL-22 (oligomer and monomer) was incubated in PBS (pH 7.4) buffer at 4°C. After centrifugation, insoluble aggregates were discarded, and supernatants were collected at different time points and subjected to SDS-PAGE analysis. The intensity of oligomeric IL-22 band was gradually decreased over time, while mIL-22 exhibited a partially cleaved pattern rather than aggregated. The spontaneous cleavage of mIL-22 is likely due to the pre-inserted proteolytic sequence as shown in **Supplementary Fig. 1**. (B) After 8 days of incubation, the formation of insoluble aggregates was confirmed as a pellet by centrifugation. (C) On the last day, the remained soluble IL-22 was visualized by WB analysis using an anti-IL-22 Ab. The data showed that the total protein of O IL-22 was significantly reduced compared to M form.

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Supplementary Figure 4

Trx-IL-22 oligomer was assembled by disulfide bonds. SDS-PAGE and WB data showed that the residual dimeric IL-22 (peak A) under typical reducing conditions (100 mM DTT, **Fig. 1F**) was remarkably reduced by adding 50 mM TCEP, a powerful reducing agent.

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Supplementary Figure 5

SDS-PAGE analysis of cleaved mIL-22 depending on EK cleavage reaction time. (A) SDS-PAGE and WB data showed uncut control of Trx-IL-22 before enterokinase treatment. (B) During the EK cleavage reaction (1 –4 h), mIL-22 was found as an independent form without Trx tag because of the specific cleavage at DDDDK site located between Trx tag and IL-22.

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Supplementary Figure 6

To assess the effects of IL-22 alone, we treated mice with 1 μg of IL-22 every other day starting at the day of fresh water administration for 5 times. (A) Experiment scheme of mIL-22 administration during fresh water administration. (B) The change of body weight percentage in each group. (C) Typical colon image and mean colon length. (D) Inflammatory cytokines and chemokines were measured by ELISA in colon. Data are expressed as mean ± SD, n=3 per group.

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