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Generation of highly stable IL-18 based on a ligand–receptor complex structure

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Abstract

Human interleukin-18 (hIL-18), initially cloned as an IFN-γ-inducing factor, has a key role in many inflammatory diseases. We have previously developed a high production system for correctly folded active hIL-18 protein, leading to the revelation of the 3D-structure and the receptor binding mode. These findings can strongly indicate the experimental and medical applications of IL-18; however, the recombinant protein is prone to be inactivated forming multimers. Recently, therapeutic approaches using recombinant IL-18 have shown the effectiveness for treatment of cancer; indicating the necessity of a more stable protein for therapy with intertrial reliability. Here we have generated a highly stable hIL-18 with replacement of cysteine by serine based on the tertiary structure and the binding mechanism, retaining the biological activity. Similar rational designs can be applied to develop new therapeutic molecules of other cytokines.

Keywords: IL-18; Mutant; Cysteine; Stability; 3D-structure

Human interleukin-18 (hIL-18), initially cloned as an IFN-γ-inducing factor secreted by macrophages or Kupffer cells, strongly augments the production of IFN-γ both in natural killer cells and T cells; having a key role in many inflammatory diseases including allergy and autoimmune diseases [1–3]. We previously developed a high production system of correctly folded active hIL-18 protein, and it enabled us to determine both the 3D-structure and the molecular mechanism of the receptor binding mode [4,5]. This production method and the molecular mechanisms can strongly assist in the experimental and medical applications of IL-18; however, one of the most common problems of recombinant protein usage for experiments and medicine is the inactivation of the protein, usually forming aggregates [6]. Recently, therapeutic approaches using recombinant IL-18 have been examined for treatment of cancer including a clinical trial in humans; indicating the necessity for a more stable form that will allow therapy to be undertaken with intertrial reliability [7–9].

Here, we describe the generation of highly stable hIL-18 based on the tertiary structure and the binding mechanism. The mutant protein with replacement of cysteine by serine showed marked antioxidative stability without formation of the oligomers, and there was no reduction in biological activity.

Materials and methods

Vector construction and protein expression. Construction of the expression vector, expression, and purification of wild type hIL-18 protein were carried out as described previously [4]. Briefly, the coding region for mature hIL-18 (157 residues) with FactorXa cleavage site just before the hIL-18 sequence was amplified by PCR and the amplified product was cloned into the pGEX-4T-1 vector (Pharmacia). BL21 (DE3) (Novagen) was transformed by the vector, and protein expression was performed as follows: the colony with the highest expression level was cultivated overnight in 200 ml of the LB medium with 100 μg/ml ampicillin. The culture was transferred into 2 L of the LB medium with 100 μg/ml ampicillin and then incubated at 37 °C until the OD600 = 0.45; it was then cooled to 25 °C. IPTG (final concentration 1 mM) was added to the medium when OD600 = 0.5. The culture was further incubated at 25 °C for 5 h.

A bacterial cell pellet was resuspended in lysis buffer (50 mM Tris–HCl, pH 8.0, 400 mM KCl, 10 mM of 2-mercaptoethanol (2ME), and

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1 mM EDTA) with 1 mM PefaBloc (Roche), lysed by sonication, and centrifuged. The clear lysate was applied onto a GST affinity column (Pharmacia) and the column was then washed. The captured fusion protein was eluted with elution buffer (50 mM Tris–HCl, pH 8.0, and 10 mM glutathione). The protein-containing fractions were concentrated and cleaved by bovine factor-Xa (Funakoshi) at a ratio of 1% (w/w) at 4 °C. The mature hIL-18 protein was isolated using Sephacryl S-100 26/60 (Pharmacia). The fractions were then stored at 4 °C until further experiments.

Oligomerization assay of the wild protein. Oligomerization assay of the protein using wild type protein was carried out in a sealed siliconized Eppendorf tube. The protein solution (400 ng/μl) in phosphate-buffered saline (NaCl 137 mM, Na_2HPO_4 8.10 mM, KCl 2.68 mM, and KH_2PO_4 1.47 mM, pH 7.0) was agitated at 150 rpm at 37 °C for 12 h in the incubator. The sample solution was mixed with 5* concentrated SDS–sample buffer (final concentration 2% (w/v) SDS, 10% (v/v) glycerol, 0.002% (w/v) bromophenol blue, 62.5 mM Tris–HCl, pH 6.8, and with/without 5% (v/v) of 2ME) and boiled for 5 min. The samples were electrophoresed on SDS–PAGE (10–20% gradient gel) and visualized by Coomassie blue staining.

Structural analyses of hIL-18 and the receptor complex. Multiple sequence alignments of the IL-18 s were performed by ClustalW with BLOSUM matrix [10]. The structure of the hIL-18 (PDB code: 1J0S) was determined by us using nuclear magnetic resonance (NMR); and here we used the modeled structure of the hIL-18:hIL18R α complex for structural analyses [5]. Structural rendering was performed on RasMol software [11].

Production and analyses of the mutant proteins. Site-directed mutagenesis of the hIL-18 gene was performed using GeneEditor in vitro Site-Directed Mutagenesis System (Promega) according to the manufacturer's instructions. Four different primers were designed for mutations to serine at C38, C68, C76, and C127, respectively. The primer sequences were: C38S: 5′-GACTGATTCTGACTCTAGAGATAATG CACC-3′, C68S: 5′-CTATCTCTGTGAAGT CTGAGAAAATTTCA ACTC-3′, C76S: 5′-GAAAATTTCAACTCTCTCCTCTGA GAACA AAA TTATTTCC-3′, (C68S/C78S for IL-18-AS: 5′-GTAACTAT CTCTGTGAAGTCTGAGAAAATTTCAACTCTCTCCTCTGAG AACAAAATTATTTCC-3′), and C127S: 5′-GATACTTTCTAG CTTCTGAAAAAGAGAGAG-3′ All the plasmid sequences harboring respective mutations were confirmed bidirectionally. Expression, purification, and the oligomerization assays were performed the same as those for wild type protein.

Biological activity assay. A biological activity assay based on IFN-γ induction was carried out as previously described [12]. Briefly, human myelomonocytic KG-1 cells were grown in the culture medium consisting of RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, L-glutamine (2 mmol/L), penicillin (100 U/mL) and streptomycin (100 μg/mL). KG-1 cells (3.0×10^5 cells) were cultured in the presence of 0.1–50.0 ng/mL of recombinant hIL-18 for 24 h in a volume of 0.2 ml at 37 °C in a humidified atmosphere containing 5% CO_2. The culture supernatant was centrifuged to remove cells and stored at −80 °C until assay was performed. IFN-γ concentration was measured by fluorometric microvolume assay technology using FMAT 8100HTS system (Applied Biosystems).

Results

Oligomerization assay of wild type IL-18

On SDS–PAGE, wild type protein showed a marked oligomerization pattern after aeration by agitation (Fig. 1). However, the oligomerization pattern completely disappeared in the presence of 2ME; indicating that it was mainly due to the intermolecular disulfide bonds among the cysteine residues of hIL-18.

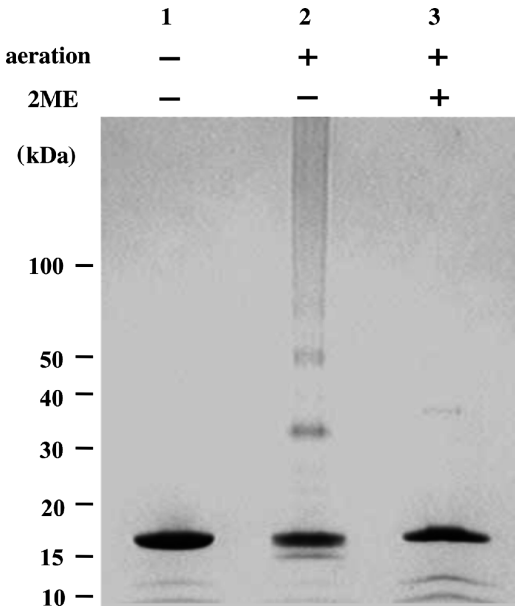

Fig. 1. Oligomerization assay of the wild type IL-18. Electrophoresis was carried on SDS–PAGE (10–20% gradient gel). (1) Before aeration without 2ME, (2) after aeration without 2ME, and (3) after aeration with 2ME. Four micrograms of each protein was loaded. The wild type protein shows a marked oligomerization pattern after aeration, but the pattern disappeared with 2ME.

Structural analyses of hIL-18 and the molecular mechanisms of receptor binding

Alignment of the amino acid sequences of the IL-18 proteins showed marked similarities among the different species. C76 and C127 are conserved among all the species, while C38 and C68 were replaced by the other residues in several species (Fig. 2). Conservation among different species usually indicates the importance of the conserved residues for the structure or activity; but conservation itself cannot show the positions of the residues in the 3D-structure associated with intra- or inter-molecular disulfide bonds.

The 3D-structure of hIL-18 determined by NMR clearly showed an absence of intramolecular disulfide bonds, and cysteine residues existing on the surface of the structure suggested a possible role for intermolecular disulfide bonds in the oligomerization (Fig. 3A). The atomic interactions among the cysteine residues and the other residues of hIL-18, and also the positions of the cysteine residues on the complex structure with hIL-18R α suggested that the replacement of the four cysteine residues by other types of amino acids, especially the conservative residue, serine, does not collapse the 3D-structure of hIL-18; retaining the capacity to bind to the receptor (Figs. 3A and B). According to structural analyses, five mutant proteins were designed (Table 1).

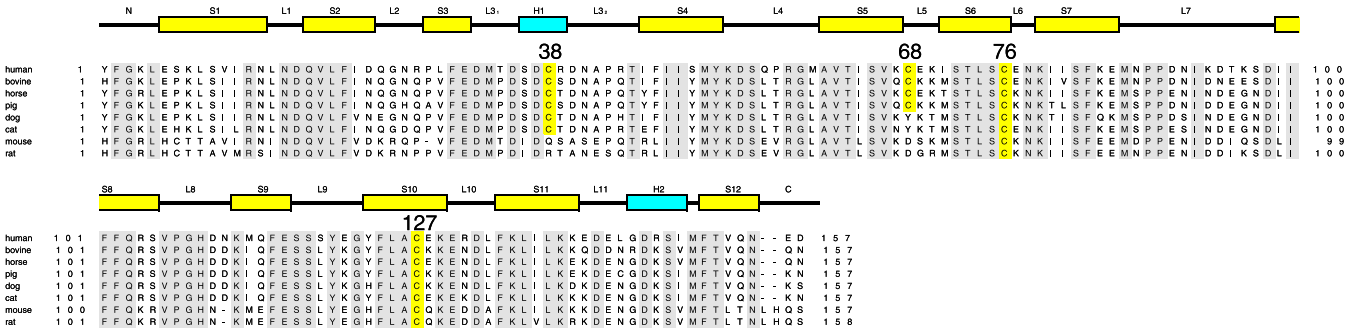

Fig. 2. Alignment of the amino acid sequences of IL-18 proteins. Completely conserved residues among the eight species are boxed in gray. The cysteine residues are boxed in yellow. The numbers of the cysteine residues are indicated as a human sequence. The secondary structure for human IL-18 previously determined by us is indicated over the sequences as the yellow (S; β-strand) and the blue (H; helix) boxes. The intermediate loop regions are indicated as L. C76 and C127 are conserved among all the species, while C38 and C68 were replaced by other residues in several species.

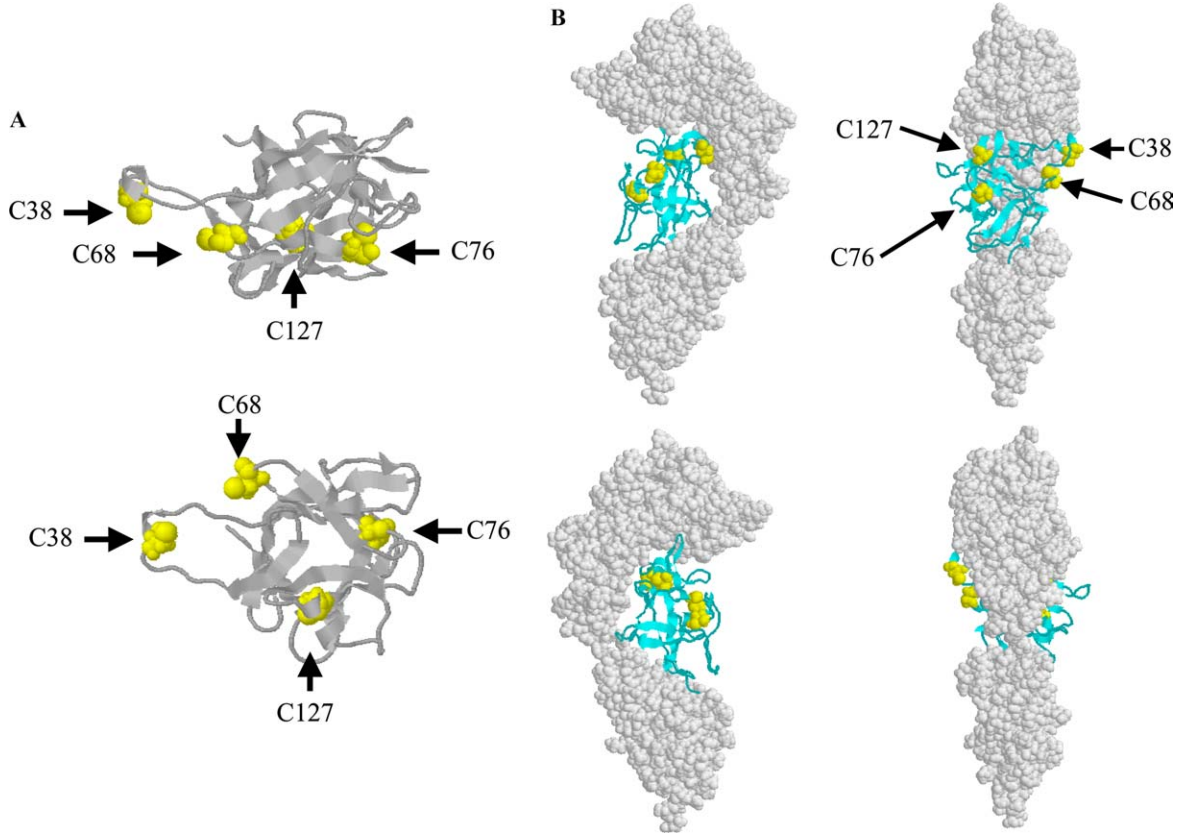

Fig. 3. Structure of the human IL-18 protein and the complex structure of human IL-18 and human IL-18 receptor α. (A) Structure of the human IL-18. The overall structure is shown as a ribbon model, and the four cysteine residues are shown as spacefill representation in yellow. The top figure and the bottom figure are tilted at 90° to each other. The four cysteine residues do not form any sulfide bonds and exist on the surface of the structure. (B) The complex structure of human IL-18 and human IL-18 receptor α. The overall structure of hIL-18 is shown as a ribbon model in cyan, and the four cysteine residues are shown as spacefill representations in yellow. hIL-18R α is shown as spacefill representation in white. The four cysteine residues of hIL-18 do not have any direct interaction with hIL-18R α; suggesting little influence on the binding.

Oligomerization assay of the IL-18 mutants

On SDS–PAGE, the mutants harboring one cysteine residue on the surface showed dimerization after the aeration procedure, but with different degrees (Figs. 4A and B). The findings indicate that all the cysteine residues of hIL-18 associated with the oligomerization phenomenon but to different extents. On the other hand, the IL-18-AS protein did not show any oligomerization pattern even after the aeration procedure (Fig. 5); indicating that the IL-18-AS protein can exist as a monomer in oxidative conditions.

Table 1
Amino acid composition of wild and mutant proteins

Position	38	68	76	127
Wild	Cys	Cys	Cys	Cys
C38	Cys	Ser	Ser	Ser
C68	Ser	Cys	Ser	Ser
C76	Ser	Ser	Cys	Ser
C127	Ser	Ser	Ser	Cys
AS	Ser	Ser	Ser	Ser

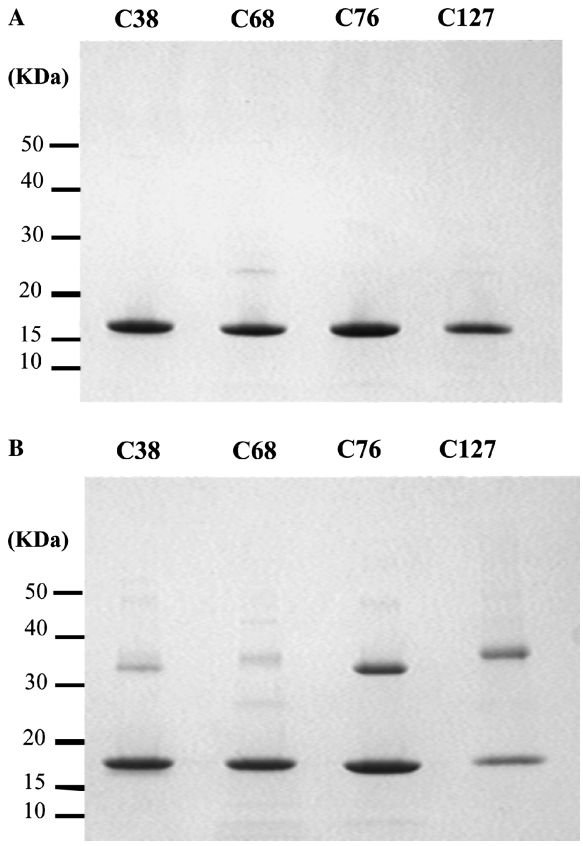

Fig. 4. Dimerization assay of the IL-18 mutants harboring one cysteine residue on the surface. Electrophoresis was carried out on SDS–PAGE (10–20% gradient gel). (A) Before aeration without 2ME, (B) and after aeration without 2ME. Four micrograms of each protein was loaded. All the mutants showed dimerization after aeration, indicating that all the cysteine residues of IL-18 associated with the oligomerization phenomenon.

Biological activity of wild and mutant protein, IL-18-AS

The biological activities of the wild type and IL-18-AS before aeration were compared. IFN-γ induction by different concentrations of the two IL-18 proteins showed no significant differences before oxidation (Fig. 6). The biological activities after aeration showed a marked reduction in the wild type protein; resulting in about five to ten times lower IFN-γ induction than that before oxidation between 1 and 10 ng/ml. However, IL-18-AS did not show any significant reduction even after extensive aeration (Fig. 6). These findings indicate

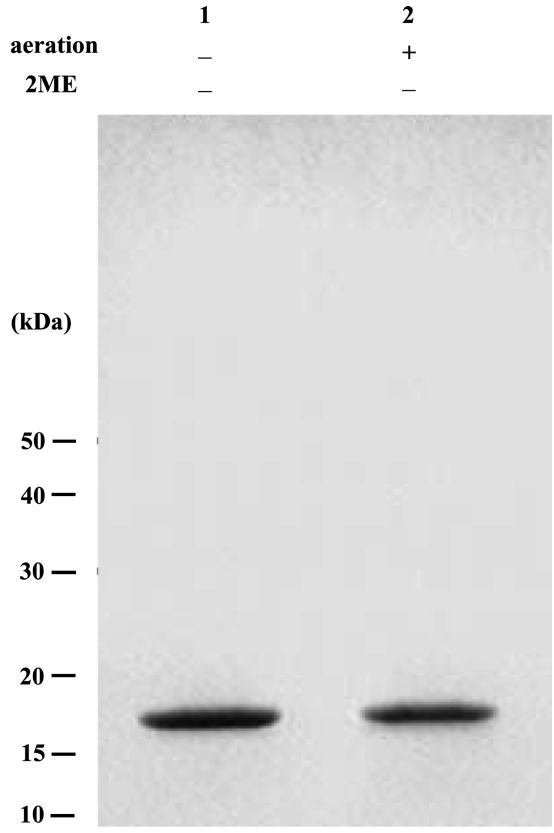

Fig. 5. Oligomerization assay of IL-18-AS. Electrophoresis was carried on SDS–PAGE (10–20% gradient gel). (1) Before aeration without 2ME, (2) after aeration without 2ME. Four micrograms of each protein was loaded. The IL-18-AS protein did not show any oligomerization pattern.

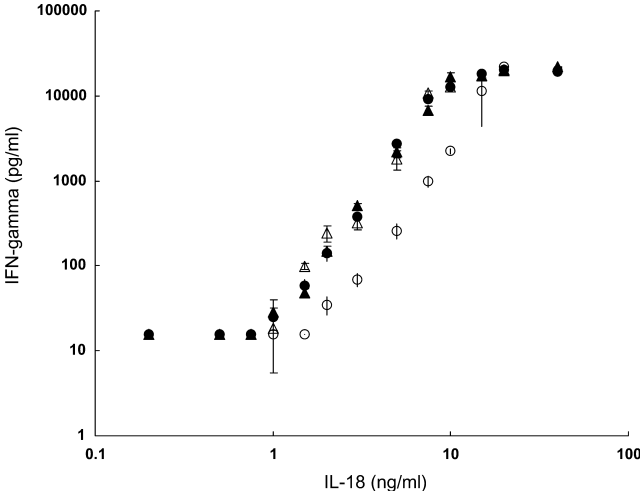

Fig. 6. IFN-γ induction (pg/ml) by the wild type and the IL-18-AS before and after oxidation. Mean values of triplicate IFN-γ induction assays are shown with standard deviation. Solid circle, hIL-18-wild before oxidation; open circle, hIL-18-wild after oxidation; solid triangle, hIL-18-AS before oxidation; and open triangle, hIL-18-AS after oxidation. IL-18-AS showed the same activity as that of the wild type even after oxidation, while wild type protein showed significant reduction in the activity after oxidation.

that the newly generated mutant, IL-18-AS, is highly stable against oxidative conditions; retaining the equivalent biological activity.

Discussion

Our method for the purification of hIL-18 shows that recombinant hIL-18 exist almost as a monomer in solution [4]. However, oligomerization and inactivation of IL-18 have been reported [13–15] and another of our studies also indicated that a small fraction of the purified protein existed as dimer or trimer even in an intensively reduced condition; suggesting that the IL-18 produced should be partially inactive [Kato et al. unpublished data]. However, the precise mechanisms of the inactivation have not yet been clarified.

Kikkawa et al. [14] speculated that one of the mechanisms of the inactivation should be misfolding; by the loss of the specific covalent intramolecular required for potent IFN-γ inducing function of IL-18, but the 3D-structure determined by us revealed that there are no intramolecular disulfide bonds in hIL-18 (Fig. 3). Further, the side chains of all the cysteine residues exist on the surface of the protein making it possible to be accessible by each other. From these observations, we can speculate that the oligomerization of IL-18 can be done using these free sulfatides on the molecular surface, and that the activity should be lost.

We have demonstrated here that wild type IL-18 forms extensive oligomers in oxidative conditions, and that the IFN-γ inducing activity was significantly reduced. The oligomerization mechanism is mediated by the intermolecular disulfide bonds among the four cysteine residues in IL-18 polypeptide, namely C38, C68, C76, and C127. To obtain an antioxidative stable hIL-18, we conservatively mutated all the cysteine residues to serine; and as the 3D-structure analyses predicted (Fig. 3), the resulting mutant protein, IL-18-AS, showed high stability retaining biological activity even after oxidation (Fig. 6).

In human fibroblast growth factor (hFGF), replacement of two of the four cysteine residues by serine (C70S and C88S) could improve instability; retaining the biological activities, but replaced the other two cysteine residues (C26S and C93S) resulting in a marked reduction in activity [16,17]. Biochemical analyses suggested that two cysteine residues (C26 and C93) form an intramolecular disulfide bond [17]. However, the structural determination of hFGF has revealed that C26 and C93 have no intramolecular sulfide bond with their side chains buried inside the molecule; indicating that the atomic interactions among the other residues will be important for the activity, while the side chains of C70 and C88 are exposed to the solvent without any disulfide bonds [18]. Moreover, the recent structure determination of the complex involving hFGF:FGF receptors has also revealed that C26 and C93 exist adjacent to the interface between ligand and receptor, while C70 and C88 are completely free from the interface of the receptor, similar to those observed in hIL-18 and the receptor [5,18].

Using a protein as a therapeutic agent requires not only production of a sufficient quantity of homogeneous protein, but also a stable formulation suitable for storage and delivery. The IL-18-AS protein that we have developed here showed significant stability against oxidation; retaining the same activity with wild type protein. Although recombinant hIL-18 has been examined in the treatment of cancer [7–9], the structure-based design of the mutants should be emphasized and the stable form of cytokine can be the first step for development of a more potent cytokine with additional site-specific modifications including glycosylation or pegylation [19–22].

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