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Soluble expression of recombinant human CD137 ligand in *Escherichia coli* by co-expression of chaperones

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Abstract CD137 ligand (CD137L) is a member of the tumor-necrosis factor superfamily that binds CD137 to provide positive co-stimulatory signals for T cells activation. Co-stimulation through CD137/CD137L has become one of the promising approaches for cancer therapy. Previous reports have shown that CD137L expressed in *Escherichia coli* resulted in inclusion bodies or low yield. In this study, the effects of five different chaperone teams on the soluble expression of recombinant human CD137L protein were explored and analyzed. The poor expression of CD137L in the cytoplasm of *E. coli* was improved significantly by co-expression of chaperone GroES-GroEL-Tf. After dual induction and affinity chromatography, purified recombinant CD137L was obtained at a yield of 3 mg protein per liter with purity greater than 98% from original undetectable level. Additionally, the purified recombinant CD137L could bind CD137-positive cells in a dose-dependent manner, markedly promote the growth of activated mice T cells, and elevate the release of IL-2. The present work provides an effective system for soluble expression of functional human co-stimulatory molecule CD137L, which will facilitate the clinical developments of recombinant protein drugs.

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Introduction

In the immune responses mounted by the body in response to pathogenic organisms and cancer, the cellular immune responses mediated by T cells, in particular by $CD8^+$ T cells, play a central role. For full T cell activation, the "two-signal hypothesis" explains that an antigen-presenting cell must provide two independent signals. Signal 1 occurs through the T-cell receptor: MHC: antigen complex while the signal 2 is provided through co-stimulation. A number of co-stimulatory molecules have been identified that function to further enhance and extend the activation of T cells. CD28 is the most prominent co-stimulatory receptor for naive T cells by providing both a qualitatively distinct second signal and amplifying the transcriptional effects of T-cell receptor triggering. Another co-stimulatory molecule CD137L (also called 4-1BBL) shares the similar ability with CD28 to co-stimulate expansion and development of effector function in memory T cells and exhibits great therapeutic potential [1].

CD137L is a member of the tumor-necrosis factor (TNF) superfamily that combines CD137 (also called 4-1BB), a member of the TNF receptor (TNFR) superfamily, and provides positive co-stimulatory signals for T cells activation [1]. It has been shown that the co-stimulation through CD137/CD137L could enhance the anti-tumor activity of T lymphocytes by inducing T cell proliferation, promoting cytokine production and functional maturation, and prolonging $CD8^+$ T cell survival [9]. Engagement of CD137 by agonist monoclonal antibodies (mAbs) or natural ligands has demonstrated impressive therapeutic efficacy in various

preclinical cancer and tumor models [6, 8, 16]. The phase II results of the humanized agonistic anti-CD137 mAb (designated BMS-663513) from Bristol-Myers Squibb demonstrated that it could survive in subjects with previously treated unresectable stage III or IV melanoma as long as 1 year (ClinicalTrials.gov identifier: NCT00612664). However, its safety profile was designated as safety issues. Another report also showed that multiple injections of anti-CD137 mAbs at therapeutic doses in naive mice resulted in transient toxicity and the development of a series of immunological anomalies [14]. Alternatively, a biologically active form of CD137L may have better efficacy and safety for cancer therapy.

To prepare for future large-scale production of CD137L and its clinical application, a simple, efficient, and cost-effective expression system was needed. *Escherichia coli* was chosen for use in this study. Although soluble recombinant CD137L could be obtained in *E. coli* by fusion with another highly soluble protein, such as glutathione-S-transferase (GST) [2, 17], recombinant CD137L fused with small peptide tags, such as AviTag or E tag, was produced as inclusion bodies in *E. coli* BL21 (DE3) strain [13] or low expression levels of periplasmic protein in *E. coli* HB2151 strain [7]. To reach a high yield of soluble and functional recombinant CD137L, we engineered a bacterial expression vector pET22b-CD137L consisting of the extracellular domain of human CD137L linked to a C-terminal His-tag, expressed in *E. coli* BL21 (DE3) or BL21 (DE3) strain harboring different chaperone plasmids and determined its optimal fermentation conditions in shake flasks.

Materials and methods

Animals

BABL/c male mice (provided by Qinglongshan Experimental Animal Center, Nanjing, China) were bred under pathogen-free conditions and were used for experiments at 6–10 weeks of age. Animal experiments were performed according to institutional and national guidelines.

Reagents and strains

pORF-h41BBL, which contains the full cDNA sequence of human CD137L, was obtained from InvivoGen (San Diego, CA, USA). The DNA polymerase, restriction enzyme, and T4 DNA ligase were purchased from TAKARA (Otsu, Shiga, Japan). DNA purification and DNA extraction kits were offered by Axygen Scientific Inc (Union City, CA, USA). *E. coli* Top10, *E. coli* BL21 (DE3) and pET22b vector were from Novagen (San Diego, CA, USA). Anti-CD3 (145-2C11) mAb and anti-CD28 (37.51) mAb were purchased from eBioscience (San Diego, CA, USA). All materials for protein purification were obtained from GE Healthcare (Uppsala, Sweden). The primers used in this study were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd (Shanghai, China) and other chemicals used in this study were of analytical or higher grade. For co-expression experiments, the Chaperone Plasmid Set (TAKARA) was used.

Construction of CD137L expression plasmid

Oligonucleotide primers were designed based on the DNA sequence of human CD137L gene (Gene ID: NM_003811). Forward (5′-GGAATTCCATATGGCCGTCTTCCTCGCC T-3′) and reverse (5′-CCGCTCGAGTTCCGACC TCGG TGAAG-3′) primers were exploited to introduce *Nde*I and *Xho*I restriction sites (underlined). Genes encoding residues 46–254 of CD137L was obtained by PCR amplification of pORF-h41BBL. After being digested with *Nde*I and *Xho*I, the amplified CD137L fragment was ligated into the corresponding sites of plasmid pET22b and completely sequenced. The positive recombinant plasmid, pET22b-CD137L, was then transformed with or without a chaperone plasmid into *E. coli* BL21 (DE3) for gene expression.

Expression of recombinant CD137L in *E. coli* by a two-step procedure

E. coli BL21 (DE3) harboring the pET22b-CD137L plasmid was screened in LB agar plate with 100 μg ampicillin/ml, and the *E. coli* BL21 (DE3) harboring both pET22b-CD137L plasmid and chaperone plasmid was screened with extra 20 μg chloramphenicol/ml. Single colonies from the transformed cells were used to inoculate 100 ml of LB medium supplemented with appropriate antibiotics in a 1,000-ml flask and cultured at 37°C in I26R stackable incubator shakers (New Brunswick Scientific Co., Inc, Edison, NJ) at 200 rpm. Sequential induction of chaperones and target protein was adopted due to its strong advantages in enhancement of solubility [3]. The induction of chaperone and recombinant CD137L overproduction was respectively triggered by adding different concentrations of tetracycline (1–10 ng/ml) or L-arabinose (0.5–4 mg/ml) at an OD_{600} of 0.3 and IPTG (0.1–1 mM) at an OD_{600} of 0.9. After initiation of CD137L induction, bacterial growth was carried out for different times at 15, 25, or 30°C to obtain the optimal folding environment for recombinant protein production. During cultivation, samples were taken every 2 h for analysis of cell density and the expression was terminated when the total *E. coli* proteins arrive at a stop signal. Cells were then harvested by centrifugation and the pellets were frozen at −80°C.

Purification of recombinant CD137L in *E. coli*

Samples were thawed on ice, re-suspended in 20 mM PBS (pH 7.4), and lysed by high-pressure cell-disruption systems (Constant Systems, Daventry, UK). The lysate was centrifuged for 30 min at 12,000 rpm at 4°C, and the supernatant was clarified by passage through a filter with 0.45-μm pores. The protein was purified by immobilized metal affinity chromatography (IMAC) on a Ni Sepharose high-performance column (GE healthcare) equilibrated in 20 mM PBS (pH 7.4) containing 0.2 M NaCl. The column was successively washed with this buffer containing 20 and 50 mM imidazole (10 and 5 volumes, respectively). The CD137L was eluted with 400 mM imidazole in the same buffer. The CD137L was dialyzed and concentrated with a centrifugal filter device (Millipore Corp., Bedford, MA, USA), and quantified by BCA assay (KEYGEN Biotech, China). Samples (10 μg) were mixed with sample buffer, namely 60 mM Tris-HCl buffer (pH 7.4) containing 2% SDS, 25% glycerol and 0.1% bromophenol blue with 2-mercaptoethanol, and were analyzed by 15% SDS-PAGE. The molecular weight of purified recombinant CD137L was estimated by gel filtration on a Superdex 200 5/150 GL column (GE Healthcare).

Cell ELISA assay

To test the binding force between CD137L and CD137, 96-well plates (Corning-Costar, NY, USA) were coated overnight at 37°C with A549 (a lung carcinoma cell line, which constitutively expresses CD137 [15], 5×10^3/well). After blocking with PBS/3% BSA, 1.875, 3.75, 7.5, 15, and 30 μg/ml CD137L diluted in RPMI 1640 were added in duplicate and incubated at RT for 2 h. The plate was then washed five times with 300 μl of PBS with 0.05% Tween-20 per well. After the last washing, the plate was inverted and blotted on absorbent paper to remove any residual buffer. Specifically bound CD137L was detected using an HRP-conjugated anti-His-tag and *o*-phenylenediamine dihydrochloride (OPD) (Sigma-Aldrich, St Louis, MI, USA) as the colorigenic substrate. The absorbance was measured at 490 nm using a microplate reader (BioTek Instruments Inc., Winooski, VT, USA). The absorbance value of A549 cells without CD137L (0 μg/ml) was used as reference and subtracted from all samples.

T Cells stimulation in vitro

Mice T cells were purified from single cell suspensions of mice splenocytes using EasySep Mouse T Cell Enrichment kit (StemCell Technologies, Vancouver, BC, Canada), according to the manufacturer's suggested protocol by negative selection through an EasySep Magnet (StemCell Technologies). Purified T cells were plated at 10^5 per well in RPMI 1640 with 10% FCS and 2 μg/ml soluble anti-CD28 mAb in 96-well plates, which were coated with anti-CD3 mAb at 5 μg/ml, in the presence or absence of 10, 2.5, and 0.625 μg/ml coated recombinant CD137L. After initiating the cultures for 44 h, Alamar blue (BioSource International, Camarillo, CA, USA) was added at 10% of the culture volume. Alamar blue contains an oxidation–reduction indicator that changes color in proportion to cell proliferation. After 48, 96, and 144 h of incubation, the plates were read by the microplate reader at wavelengths of 570 and 600 nm. Cell proliferation corresponds to the magnitude of dye reduction and is expressed as percent reduction.

Cytokine assay

All groups of cell culture medium supernatants were collected at 144 h and assayed by ELISA for murine IL-2, using paired antibodies in accordance with the manufacturer's instructions (eBioscience).

Results and discussion

With an increased understanding of the cellular and molecular mechanisms of T cell activation, new therapeutic strategies have emerged and have led to remarkable beneficial effects in the treatment of cancer by engaging co-stimulatory pathways (e.g., CD28:B-7, CD137:CD137L, OX40:OX40L) or blocking the engagement of inhibitory receptors (e.g., CTLA-4, PD-1, BTLA) [5]. Co-stimulatory or co-inhibitory monoclonal antibodies have been investigated extensively for their abilities to induce anti-tumor immunity and have shown great outcomes in mice and humans [12]. Although monoclonal antibodies have revolutionized the treatment of a number of malignancies, whole antibody may lead to higher costs and serious side-effects. Since the physiological ligands of these receptors have similar biological functions to antibodies but without the side-effects and toxicity of the latter, biologically active forms of ligands for these receptors can also be envisioned as potential therapeutics. However, few papers have reported a high yield of soluble and functional human CD137L until now [10].

Recombinant CD137L plasmid construction

Human CD137L is composed of a cytoplasmic domain (residues 1–25), a transmembrane domain (residues 26–48), and an extracellular domain (residues 49–254) [1]. Since the extracellular domain retains its full capacity for T cell activation, the coding sequence for residues 46–254 was amplified from plasmid pORF-h41BBL and inserted into the pET22b vector. A 6 × His-tag was fused to the C-terminus

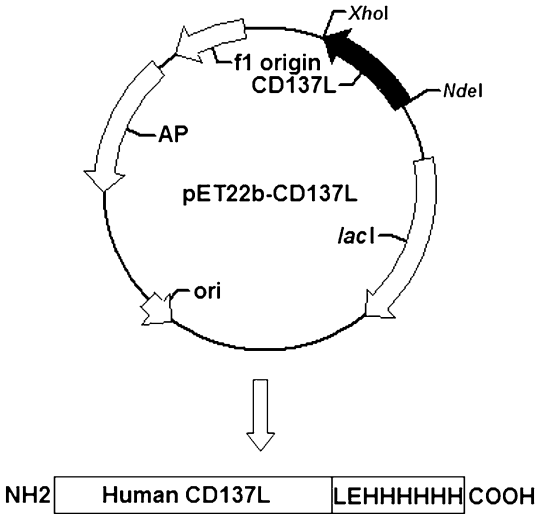

Fig. 1 Schematic representation of the expression constructs for recombinant CD137L (drawings are not to scale). Human CD137L (residues 46–254) was amplified and cloned into the pET22b vector to create the C-terminal His-tagged CD137L. Two amino acids (Leu and Glu) were incorporated between them due to the *Xho*I coding sequence

of CD137L for purification and detection purposes, and two amino acids (Leu and Glu) were incorporated between them due to the *Xho*I coding sequence (Fig. 1). Ten clones were picked. The results of restriction endonuclease digestion and sequencing analysis showed that the target fragment had been successfully inserted into the vector. The first positive clone was named pET22b-CD137L and used for the following high-level expression of soluble recombinant CD137L in a most widely used host *E. coli* BL21 (DE3).

Expression and purification of recombinant CD137L

In general, low induction temperatures or growth in minimal inducer and media tend to increase the percentage of target protein found in soluble form, but all of these factors showed no obvious effects on the expression of recombinant CD137L (data not shown). Previous studies have shown that co-expression of a target protein with molecular chaperone increases recovery of expressed proteins in the soluble fraction [11]. Therefore, optimal culture conditions for co-expression of recombinant CD137L and five different types of chaperone team from Takara's Chaperone Plasmid Set were investigated in the following study.

The initial co-expression experiment was carried out according to the manufacturer's protocol with or without chaperone plasmid pG-KJE8, pGro7, pKJE7, pTf16 or pG-Tf2, which coded DnaK-DnaJ-GrpE-GroES-GroEL, GroES-GroEL, DnaK-DnaJ-GrpE, Tf or GroES-GroEL-Tf chaperone team, respectively. Although all kinds of chaperone teams have been correctly expressed, the soluble recombinant

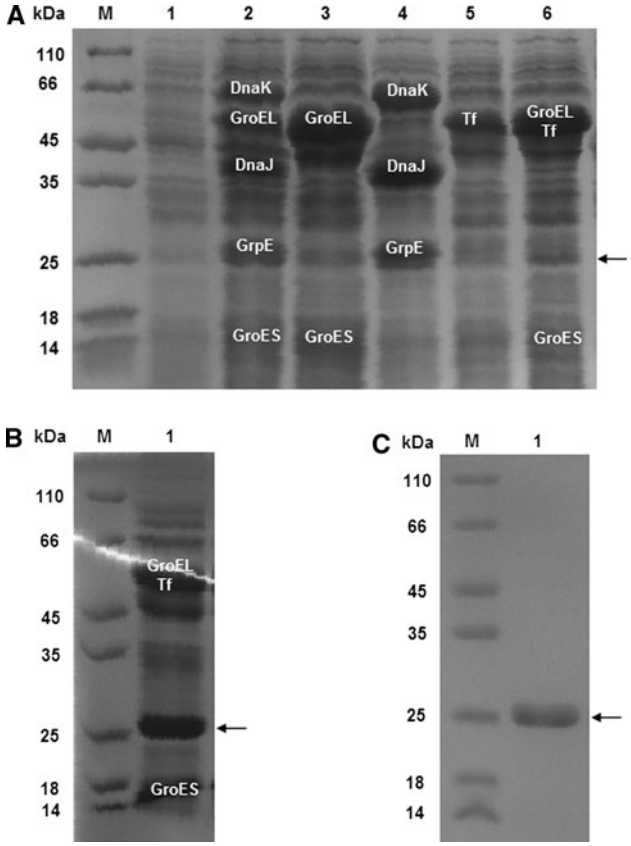

Fig. 2 SDS-PAGE analysis of recombinant CD137L. **a** Co-expression of recombinant CD137L with different chaperone teams. *Lane 1* supernatants from *E. coli* BL21 (DE3) strain, *Lane 2–6* supernatants from *E. coli* BL21 (DE3) strain harboring chaperone plasmid pG-KJE8, pGro7, pKJE7, pTf16, and pG-Tf2, respectively. Chaperone proteins were marked on the corresponding bands. **b** Optimization of the expression condition of recombinant CD137L. *Lane 1* supernatants from *E. coli* BL21 (DE3) strain harboring chaperone plasmid pG-Tf2 under the optimal expression condition. **c** SDS-PAGE analysis of the purified recombinant CD137L (3 mg purified CD137L/l culture). *Lane M* protein molecular weight marker. *Arrow* shows the target protein

CD137L was only slightly enhanced compared to the *E. coli* BL21 strain without chaperone plasmid (Fig. 2a). It is a misleading appearance that DnaK-DnaJ-GrpE-GroES-GroEL and DnaK-DnaJ-GrpE chaperone teams could significantly contribute to the soluble expression of our target protein (Fig. 2a, Lane 2, 4), because the size of chaperone GrpE is very close to recombinant CD137L, and the later purification also certified that there were no significant differences in the expression level of CD137L among cultures. After changing the timing of induction, inducer concentration, and induction period, a higher level expression of target protein was only achieved by co-expression of GroES-GroEL-Tf chaperon team under the following conditions: 0.2 mM final concentration of IPTG for target protein induction and 10 ng final concentration of tetracycline/ml for chaperone GroES-GroEL-Tf induction at 15°C for 16 h (Fig. 2b). The purity of the target protein reached more than

98% by SDS-PAGE (Fig. 2c) and a highest yield of 3 mg protein per liter was obtained. The following gel filtration confirmed that the recombinant CD137L is expressed as a trimer (data not shown). Co-expression of other chaperone teams was relatively less effective for production of soluble CD137L in *E. coli* under the conditions employed, which is reasonable since the effects of different chaperones may differ depending on the specific target protein used.

Functional assays of recombinant CD137L

Considering that solubility may not equal to activity, we next analyzed the binding activity of recombinant CD137L with CD137 using cell ELISA. As a convenient and useful technique for the relative quantitative detection of cell-surface molecular, cell ELISA has been used universally since it was initially reported [4]. Using this method, we demonstrated that recombinant CD137L produced in *E. coli* could specifically bind CD137 expressed on the A549 tumor cells in a dose-dependent manner (Fig. 3), which indirectly certified the functional activity of target protein due to a close correlation between the cell-binding activity and functional activity.

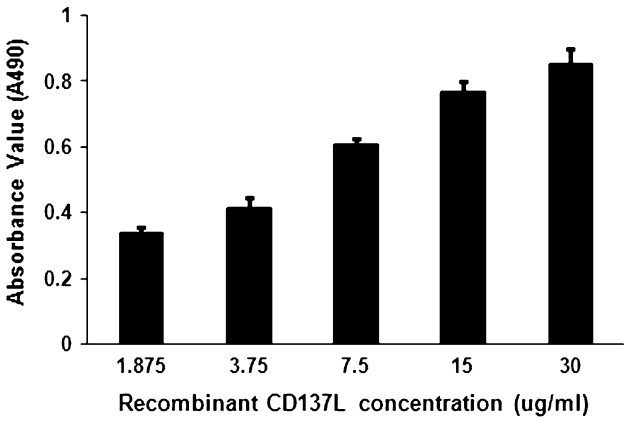

Fig. 3 ELISA analysis of the specific bioactivity binding between recombinant CD137L and A549 cells. Data shown (mean ± SD) are representative of three separate experiments

To investigate the therapeutic effect of recombinant CD137L in detail, we next measured the effect of recombinant CD137L on T cell proliferation and cytokine secretion. After T cells were stimulated with plate-bound anti-CD3 mAb, soluble anti-CD28 mAb and various doses of plate-bound recombinant CD137L, the proliferative response was evaluated by Alamar blue reduction after incubation for 48, 96, and 144 h, respectively. As shown in Fig. 4, there were no significant differences between the groups at 48 h. At 96 and 144 h, stimulation of T cells with anti-CD3 mAb and anti-CD28 mAb resulted in only 6–8% more Alamar blue reduction than the anti-CD3 mAb alone, while additional co-stimulation with recombinant CD137L at 2.5 and 10 μg/ml produced 18–28% more Alamar blue reduction. At 144 h, the level of IL-2 (435.07 ± 23.34 and 284.76 ± 12.12 pg/ml) in group of 10 and 2.5 μg/ml CD137L was significantly higher ($p < 0.05$) than that of anti-CD3 mAb and anti-CD28 mAb co-stimulated group (37.15 ± 1.21 pg/ml) (Fig. 5). These data indicate that the proliferation and the cytokine release of activated T cells from mice can be further promoted by recombinant human CD137L in vitro. It seems implausible since human CD137L shares only

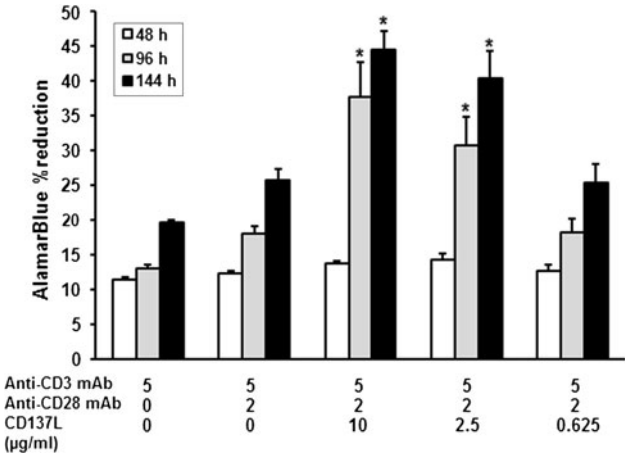

Fig. 4 Recombinant CD137L promotes proliferation of activated mice T cells. Mice T cells (10^5/well) were cultured in triplicate for 48, 96, and 144 h with anti-CD3 mAb, anti-CD28 mAb and varying doses of recombinant CD137L as shown. Proliferative responses were measured by Alamar blue assays and expressed as percent reduction of the dye. Data are expressed as mean ± SD of triplicate cultures. *$p < 0.05$ compared with anti-CD3 mAb and anti-CD28 mAb co-stimulated group at the same time point

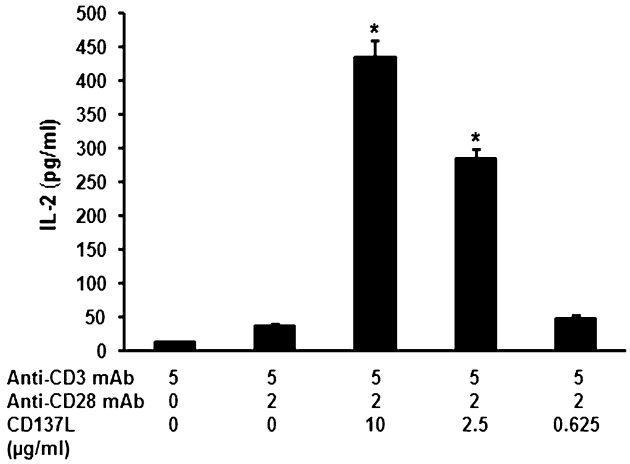

Fig. 5 IL-2 secretion from mice T cells with or without CD137L co-stimulation. The levels of IL-2 in the cell culture supernatants were analyzed by ELISA following 144 h of incubation in the absence or presence of anti-CD3 mAb, anti-CD28 mAb and varying doses of recombinant CD137L as shown. *Bars* represent mean ± SD of triplicate cultures. *$p < 0.05$ compared with anti-CD3 mAb and anti-CD28 mAb co-stimulated group

36% identity with the murine counterpart, but our study for the first time showed that they did cross-react, which indicated that future in vivo studies to characterize the recombinant human CD137L can be conducted in mice, and their cross-activities will be systematically explored and compared in our future study.

Taken together, we successfully developed a method to express soluble and functional recombinant CD137L in *E. coli* by co-expression of GroES-GroEL-Tf chaperone team and also reported its optimum expression conditions in shake flasks, which may be useful for future scale-up and possible eventual clinical development.

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Conflict of interest The authors declare that they have no conflicts of interest.

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