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RESEARCH ARTICLE

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Recombinant CD137-Fc, its synthesis, and applications for improving the immune system functions, such as tumor immunotherapy and to reduce the inflammation due to the novel coronavirus

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Abstract

CD137 (ILA/4-1BB), a member of tumor necrosis factor receptor superfamily, is one of the most important T cell costimulatory molecules. Interaction of this molecule with its ligand transmits a two-way signal that activates both T lymphocyte and antigen presenting cells. The soluble form of CD137 (sCD137) reduces the activity of its membrane isoform and is associated with T lymphocyte activation-induced cell death. Recombinant CD137-Fc may be used to treat cancers, autoimmune disorders and viral infections. It may also be useful for management of coronavirus infection. The 1276 bp DNA sequence encoded CD137-Fc recombinant protein was prepared and subcloned into lentiviral vector and expressed in transduced CHO-K1 eukaryotic cells. The sodium dodecyl sulfate–polyacrylamide gel electrophoresis, Western blot analysis, and enzyme-linked immunosorbent assay analysis results demonstrated that the expression of the 70-kDa CD137-Fc molecule was detectable without any degradation. This study helps to confirm previous research suggesting the use of this recombinant protein as a promising solution for the treatment of virus infections. CD137-Fc fusion protein could also make immunotherapy more effective for some diseases. This product is widely used in novel medical treatments, including cell-based immunotherapy such as dendritic cell, CAR T and CAR NK therapy. Its production and usage in research and treatment is noticeable also in current coronavirus disease 2019 pandemic.

KEYWORDS

autoimmune disorders, cancer immunotherapy, coronavirus inflammation, recombinant protein CD137-Fc, sCD137

1 | INTRODUCTION

CD137 (also known as 4-1BB and TNFRSF9) is a member of the tumor necrosis factor receptor family which is expressed by a variety of leukocytes, including activated T cells, natural killer cells, and inflamed vascular endothelial cells.[1] CD137 ligand (CD137L) belongs to the tumor necrosis factor (TNF) family and is mainly expressed by antigen presenting cells (APCs) as a transmembrane protein on the cell surface.[2] APC and T cell use the CD137 receptor/ligand interaction to co-stimulate T cell activity as well as cytokine production by APCs.[3]

Growing evidence also suggests that the CD137 and CD137L are involved in inflammatory diseases and pathogenesis of several diseases including cancer, autoimmune diseases, and viral infections.[4–6]

A special feature of the CD137 receptor/ligand network is the ability of reverse signaling. Since both are expressed as membrane proteins with cytoplasmic domains, ligand may also activate intracellular signaling pathways in addition to the receptor.[7,8]

This two-way signaling enables this interaction to have a great impact on the regulation of immune responses and increases the chances of using it as a therapeutic target.[9,14]

Several studies have shown that manipulation of this interaction can help to treat viral infections and malignancies as well as autoimmune disorders.[9]

The CD137-CD137L axis also plays a critical role in maintaining effective T cell immune responses that generate immunological memory. It can powerfully modulate immune responses to effectively treat a variety of immune disorders.[10] CD137-Fc acts in combination with other forms of cancer treatment, to eradicate non-immunogenic and weakly immunogenic tumors.[11]

In addition to inflammatory cytokine production, CD137 activation has been shown to induce interleukin-13 (IL-13) production in CD8 and CD4 T cells to reduce the inflammation.[10–12] Recent findings indicate the function of CD137 and its role in the immune response, which enhances both antiviral and antitumor responses, while alleviating certain autoimmune conditions.[11,13]

It has been shown that there is a soluble isoform in the TNF receptor superfamily members, which is generated by mRNA alternative splicing.[14] The soluble isoform of CD137 (sCD137) is associated with T lymphocyte activation-induced cell death.[14,15] It can attenuate the membrane bond CD137 activity, consequently reducing the stimulation of T cells. It could therefore be ideal for the treatment of autoimmune diseases and uncontrolled immune responses like cytokine storm in severe cases of COVID-19 infections.[12,15]

One of the well-known properties of sCD137 is activation of antiviral immunity to halt viral replication as well.[16] It may also reduce the inflammation of the lung caused by the COVID-19 virus and improve the condition of patients. Generally, interrupting the activation of lymphocytes by sCD137 may be helpful in reducing harmful inflammatory conditions.[15,17]

Consequently, recombinant CD137-Fc protein may have various applications in cancer immunotherapy, autoimmune disorders and viral infections.[2,4,18,19] The dual role of the 4-1BB pathway in infectious diseases and autoimmunity is well defined by some investigators.[10]

The aim of this study is to produce a stable recombinant CD137-Fc protein and propose it as an immune modulator. Preliminary studies have also been conducted for its use in the production of inflammatory dendritic cells in cancer immunotherapy and to reduce inflammation in Covid disease.

2 | MATERIALS AND METHODS

2.1 | Vector constructs and bacterial strains

"pcDNA3-EGFP" plasmid which contains hu-CD137-Fc gene (Figure S1) was kindly provided by Dr. Herbert Schwarz, National University of Singapore. Then, the huCD137-Fc gene was extracted using the Xho1 and Xba1 (Fermentas) digest sites to subclone it in a new vector (pCDH-CMV-MCS-EF1-cGFP-T2A-puro vector [Stem Cell Technology Research Center]). STBL4 strain of E. coli (Invitrogen) was used for transformation and gene cloning. Transformed bacteria, containing the PCDH vectors, were cultured in Luria-Bertani (LB) medium. Then, plasmid extraction was carried out by QIAprep spin Miniprep kit according to manufacture instructions.

2.2 | Eukaryotic cell line and culture condition

CHO-K1 (animal cell expression host) was cultured in Dulbecco's Modified Eagle's Medium (Gibco Laboratories) and HEK293T (packaging cell line) in Roswell-Park Memorial Institute (RPMI) 1640 (Gibco Laboratories), supplemented with 10% fetal bovine serum (FBS) (Gibco Laboratories), 1% penicillin/streptomycin (PAN Biotech), and 2 mM L-glutamine and incubated at 37°C in 5% CO_2. The absence of mycoplasma was confirmed for all cell lines by polymerase chain reaction (PCR).

2.3 | Subcloning of the desired fragment in the retro-viral vector (pCDH)

Bacteria containing the pCDH vector were amplified in LB with 50 μg/ml ampicillin, and plasmid extraction was performed. The combination and ligation of the purified CD137-Fc gene fragment and of the pCDH plasmid was performed using T4 ligase. These two fragments were double digested using the same restriction enzymes.

2.4 | Transfection and assembly of virus particles

PCDH vector containing the huCD137Fc gene was co-transfected along with psPAX2 (Addgene) and pMD2.G (Addgene) helper vectors into HEK 293T packaging cell line using the Lipofectamin 3000 Reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. Briefly, HEK 293T cells were cultured in six-well plates to reach 70% confluency. Then, diluted lipofectamin and DNA with Opti-MEM (Reduced-Serum) medium were mixed (1:1 ratio) and incubated for 10–15 min. Afterwards, DNA-lipid complex was added to HEK 293T cells. Transfection effect was evaluated with a fluorescence microscope after 1–3 days, since the expression vector contained GFP as a reporter gene (Figure S2).

To harvest virus particles, the supernatant of HEK293T cells was collected every 12 h for 3 days. The harvested supernatants were centrifuged at 3000 rpm for 15 min and were filtered through 0.45 μm membrane filters to exclude cell debris.

2.5 | Transduction (stable expression of recombinant protein)

Total, 5×10^4 CHO-k1 cells were seeded in each well of a 12-well plate and incubated in RPMI medium containing 10% FBS overnight. Then, the culture supernatant was removed and 100 μl of the filtered HEK 293 T cells supernatant containing the virus particles was added into each well in multiplicity of infection (MOI) of 10 with 500 μl of RPMI medium containing 5% FBS and a final concentration of 8 μg/ml polybrene, and the plates were incubated for 5 h. Afterwards, the medium volume was increased to 1 ml.

After 12 h, the culture medium containing virus particles was replaced with RPMI containing 10% FBS.

The GFP expression was evaluated 3 days later using the Nikon Multi Zoom AZ100 multi-purpose microscope to identify the transduced cells.

2.6 | Isolating the monoclonal cell populations by limiting dilution

Generating a monoclonal cell line by limiting dilution results in cell populations that are more likely to retain stable transgene expression.

The cells were detached from the stable cell pool by trypsinization, and any cell clumps were separated into individual cells by several pipetting. Approximately 10 ml of 5 cells/ml solution was prepared for each 96-well plate. Hundred microliters of cell suspension was transferred into each well of a 96-well plate to seed the plate at an average density of 0.5 cells/well. Thus, it was ensured that some wells received a single cell, while the chances of wells receiving more than one cell were minimized.

The cells remained intact for 7–14 days in the incubator. Seven days later, the wells were daily scanned for cell growth. The cells were transferred to a larger culture dish before they became over-confluent.

Following clonal expansion, enzyme-linked immunosorbent assay (ELISA) and western blot were performed to screen clones with high CD137-Fc protein expression.

2.7 | Investigation of recombinant protein expression

2.7.1 | Real time PCR assay

CD137-Fc messenger RNA (mRNA) expression in various clones was detected using reverse transcription-PCR. Briefly, total RNA was extracted using TRIZOL (Invitrogen) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized using the cDNA synthesis kit (Primescript RT reagent kit perfect real time; Takara). The expression levels of CD137L mRNA were compared to HPRT, and was defined as the $2^{-\Delta\Delta Ct}$.

2.7.2 | Enzyme-linked immunosorbent assay (ELISA)

The concentration of CD137-Fc protein in supernatants of transducted-CHO-K1 cells was measured using sandwich ELISA method. Hence, ELISA well plates were coated with anti-His Tag antibody (Abcam; ab9108). Moreover, a horseradish peroxidase (HRP)-conjugated anti-immunoglobulin G (IgG) (Sigma-Aldrich) was used as the detection antibody. Finally, yellow-colored product absorbance was read out at 450 nm wavelength by an ELISA reader (Biotek). Samples were assayed in triplicate within each experiment.

2.7.3 | Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blot assay

Western Blot assay was performed to confirm recombinant protein expression. A total of 10% polyacrylamide gel (Merck) was used to separate reaction mixtures. Coomassie Brilliant blue (Sigma-Aldrich) was also used for staining. Separated proteins were then transferred to polyvinylidene difluoride. Subsequently, the membrane was incubated

overnight with antibodies as follows: HRP-conjugated anti His-Tag antibody (Sigma-Aldrich) or HRP-conjugated anti-Human IgG (Sigma-Aldrich). Finally, the membrane was incubated and stained with ECL western blot analysis substrate kit (Cyto Matin Gene).

2.8 | Protein purification

Approximate concentration of the released CD137-Fc was investigated in the selected clones by Bradford protein assay kit, after which the supernatant containing sufficient concentration of the CD137-Fc was filtered (0.22 mm filter) and run over the affinity column containing the Ni-NTA resin. The column was then washed with wash buffers (Table 1) and the protein was eluted by increasing the concentration of imidazole to 250 mM. The eluted CD137-Fc protein was dialyzed and the concentration of purified sCD137-Fc was determined by the spectrophotometer.

2.9 | Assessment of the recombinant protein applications

One of the most effective uses of this fusion protein is the production of dendritic cells, a vital cell in cancer immunotherapy. The interaction between the coated CD137-Fc and CD137L on the surface of the monocytes induces cell maturation leading to DC characteristics, known as CD137L-DC, which are similar to the inflammatory dendritic cells in the body.

2.9.1 | Evaluation of endocytosis of CD137L-dendritic cells

Monocytes were induced to become Immature CD137L-DC after 5 days of exposure to CD137-Fc protein. Immature common DCs were also induced after 6 days of exposure of blood derived monocytes to IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF). The maturation factor (1 μg/ml Toll-like receptor 7/8 agonist coupled to 50 ng/ml interferon γ) was added on Day 6 in both groups.

The endocytosis activity of immature and mature dendritic cells was assessed by the uptake of FITC-dextran. (FD40s; Sigma-Aldrcih). CD137L-DC and common DC (induced by IL-4 and GM-CSF) were cultured in the complete culture medium containing FITC-dextran at a concentration of 0.5 mg/ml and incubated for 1 hour at 5% CO_2 and 37°C. The control cells were incubated in 4°C. After the incubation time, the cells were washed and the uptake of FITC-dextran was analyzed by flow cytometry.

2.9.2 | Evaluation of cytokine release of CD137L-dendritic cells

CD137L-DC and common DC were co-cultured separately with allogenic T cells at ratio 1:3 of DCs:T cell for 12 h in the complete culture medium at 37°C in 5% CO2. The IL-12 P70 concentration in the cultured cell supernatants were determined using ELISA kits (R&D System) according to the manufacturer's instructions.

2.9.3 | Evaluation of the CD137-Fc protein effect on COVID-19 samples

Given that the main problem with SARS-CoV-2 is inflammation due to innate immunity and cytokine release syndrome, leading to severe pneumonia, an ex vivo assay was designed to investigate the effect of our conjugated protein (CD137-Fc) on inflammatory cytokine levels in COVID-19 samples. Sampling was performed on five adult inpatients, 10 days after the onset of the disease symptoms. Their infection with SARS-CoV-2 was confirmed by real-time PCR.

In brief, leukocyte-rich plasma was cultivated in the RPMI culture medium with and without conjugated protein (CD137-Fc) in six-well plates for 16 h. The IL-6 and IL-8 levels as inflammatory cytokines were measured using the ELISA kits (R&D systems) according to the manufacturer´s protocol. Optical absorbance at 450 nm was measured using an ELISA plate reader (Multiskan; Labsystems Thermo Fisher Scientific). All experiments were done in triplicates.

2.10 | Statistical analysis

All experiments were repeated for at least three times. Data is expressed here as mean ± *SEM* of the obtained

TABLE 1 Buffers used in the purification column

Buffers	Components
Wash buffer 1	50 mM Tris, 300 mM NaCl, 60 mM imidazole (pH 7.9)
Wash buffer 2	50 mM Tris, 300 mM NaCl, 80 mM imidazole (pH 7.9)
Elution buffer	50 mM Tris, 300 mM NaCl, 250 mM imidazole (pH 7.9)

results. Statistical comparisons were evaluated by one-way analysis of variances using GraphPad Prism software (GraphPad PRISM V 8.0).

3 | RESULT

3.1 | Verifying the CD137-Fc fragment gene and addition of the appropriate restriction sites

The accuracy of the primary plasmid (donated vector) was confirmed by enzymatic digestion with XhoI and XbaI restriction enzymes. Enzymatic digestion resulting in the release of a 1276 bp recombinant gene fragment (Figure 1A).

Conventional PCR was performed to add the recognition sites of XbaI and EcoRI restriction enzymes before cloning (Figure 1B).

3.2 | Subcloning of the gene fragment into the viral vector (pCDH)

As shown in Figure 2, the pCDH size on 1% agarose gel was 8189 bp (Figure 2A). The electrophoresis band of double digested gene fragment was seen as a single band and without any smear at the expected size (Figure 2B). The 1500 bp PCR product with vector specific primers indicates the presence of plasmid containing the desired fragment (Figure 2C) (Figures S3 and S4).

3.3 | Colony PCR confirmed the existence of CD137-Fc fragment in the vector

The STBL4 E. coli strain was transformed with the produced plasmid (CD137-Fc + pCDH). The colonies that grew in Ampicillin-containing LB Agar were likely to contain new recombinant plasmids.

Colony PCR was performed using the vector-specific primers (Table 2) to confirm the presence of CD137-Fc fragment in the vector (Figure S3). As illustrated in Figure 2C, all clones had the recombinant plasmids containing the desired gene fragment.

3.4 | Double digestion of pCDH vector with Xba1 and EcoR1 and sequencing of the resulted DNA fragment

The accuracy of the cloned sequence with a length of 1296 bp was confirmed through the removal of the CD137-Fc fragment by double digestion with the same restriction enzymes used for the plasmid construction (Figure 3A)

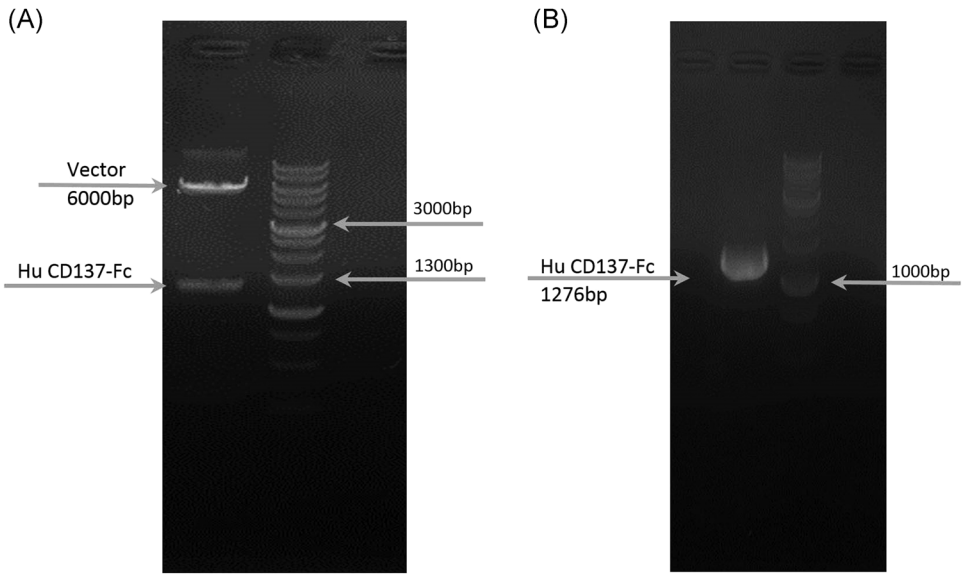

FIGURE 1 DNA construct electrophoresis. (A) Result of double digestion with XhoI and XbaI restriction enzymes: release of the desired recombinant gene fragment. (B) Before subcloning, the conventional PCR was carried out with the designed primers to multiply the recombinant DNA and add the XbaI and EcoRI restriction enzymes recognition sites to the ends of the gene fragment. PCR, polymerase chain reaction

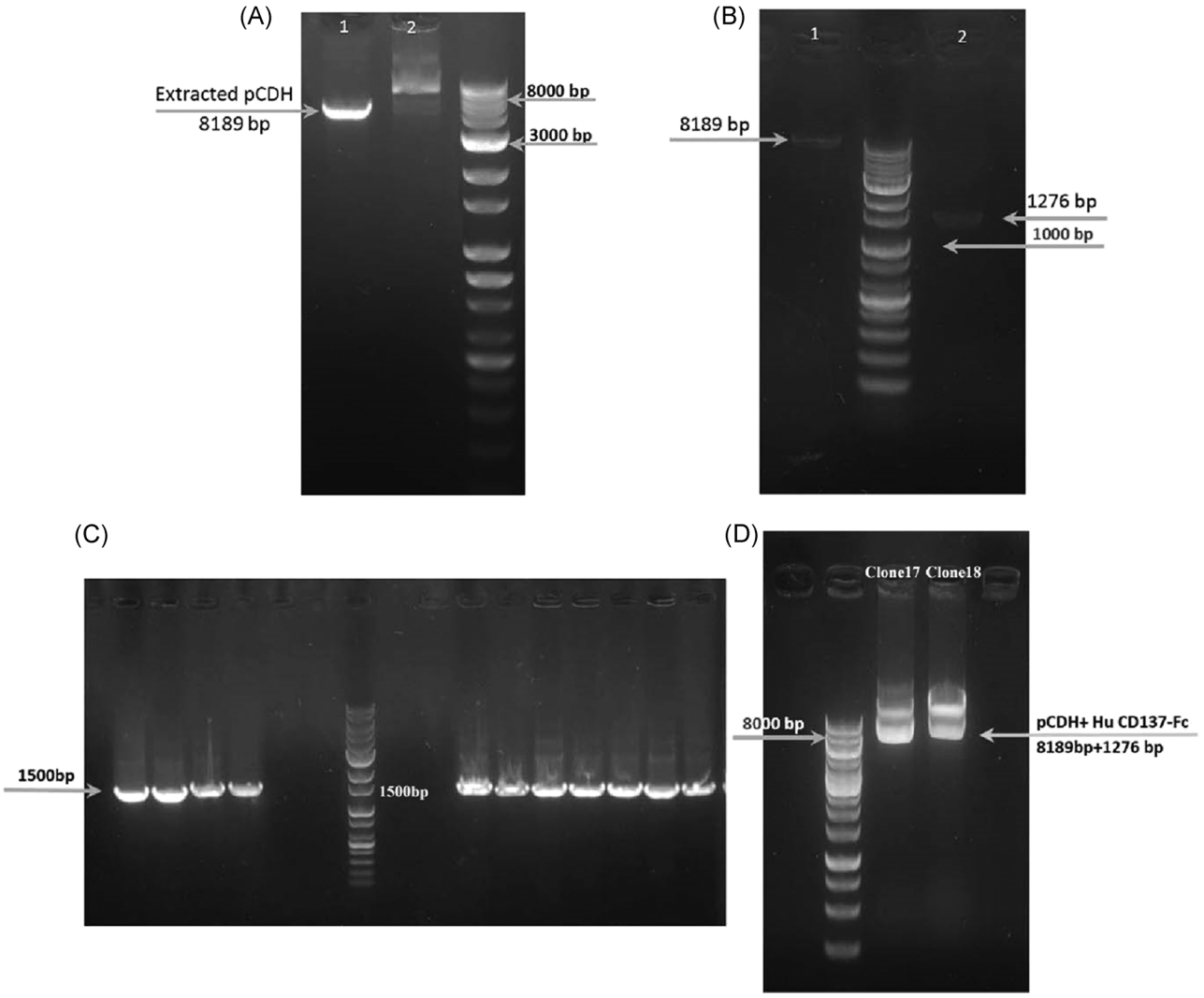

FIGURE 2 Subcloning of the CD137-Fc fragment gene. (A) The pCDH plasmid was digested by the XbaI and EcoRI restriction enzymes and cleaned. Two microliters of the nondigested circular plasmid (lane 1) and the linearized plasmid (lane 2) were then electrophoresed on 1% agarose gel. (B) The bands show the result of the pCDH (lane 1) and CD137-Fc fragment (lane 2) cutting by EcoRI and XbaI enzymes, purified by column (1 μl of each product was used for electrophoresis on 1% agarose gel). (C) Electrophoresis of colony PCR product on 1% agarose gel (1500 bp PCR product length with backbone specific primers) the lines show the results of different colonies. (D) Plasmid extraction; Two colonies (17 & 18) were selected and expanded, followed by plasmid extraction. PCR, polymerase chain reaction

Plasmid sequencing was also done from both sides with vector-specific forward and reverse primers (Table 2) (Figure 3B).

3.5 | Viral vector production (lenti virus packaging)

After extraction of the pCDH plasmid containing the CD137-Fc gene fragment, it was transfected into the HEK293T cell line along with pAX and pMD2 plasmid using the Lipofectamine3000 reagent. The size of pAX and pMD2 plasmids was 10073 and 5824 bp, respectively. The expression of GFP protein showed the creation of lentiviral vectors (Figure 4).

3.6 | Transduction of CHO-K1 cell line

The produced viruses after titration, were transduced into the CHO-K1 cell line with MOI = 10 (Figure 5).

TABLE 2 Primers sequences which were used for cloning and real time PCR and the product length

	Primer	Sequences	Product length
CD137-Fc	Forward	5′-ATGGGAAACAGTTGTTACAAC-3′	1276 bp
	Reverse	5′-TTTACTCGGAGACAGGGAG-3′	
HPRT	Forward	5′-CCTGGCGTCGTGATTAGTG-3′	125 bp
	Reverse	5′-TCAGTCCTGTCCATAATTAGTCC-3′	
pCDH backbone primer	CMV-F	5′-AATGGGCGGTAGGCGTGTA-3′	1500 bp
	EF2-R	5′-GGACTGTGGGCGATGTGC-3′	

Note: The conventional PCR was carried out with the designed primer pair (CD137-Fc forward and reverse) to multiply the recombinant DNA and to adding the recognition sites of the XbaI and EcoRI restriction enzymes. HPRT primer pair is used as internal control for real time PCR. The cloned plasmid was sequenced using the vector specific primers, CMV-F and EF2-R, and blasted in the NCBI GenBank to confirm the presence of recombinant fragment inside the plasmid.

Abbreviation: PCR, polymerase chain reaction.

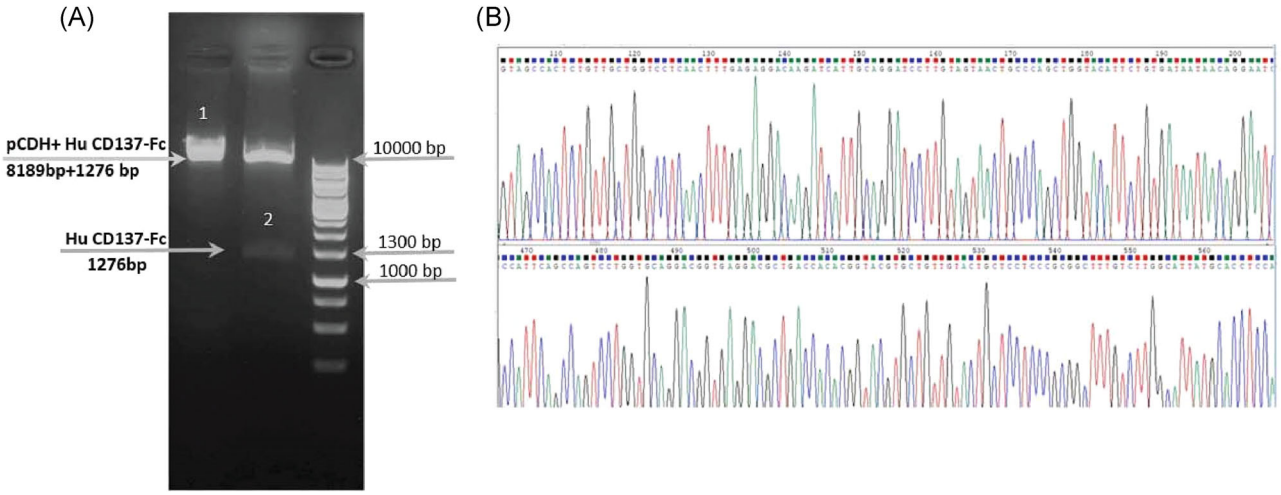

FIGURE 3 Confirmation of cloning. (A) After double digestion of the pCDH plasmid with two restriction enzymes (XbaI and EcoRI) the 1276 bp fragment was removed. (B) The cloned plasmid was sequenced using the vector (pCDH) specific primers, CMV-F and EF2-R, and blasted in GenBank (http://www.ncbi.nlm.nih.gov/) using blastn searching tools to confirm identity

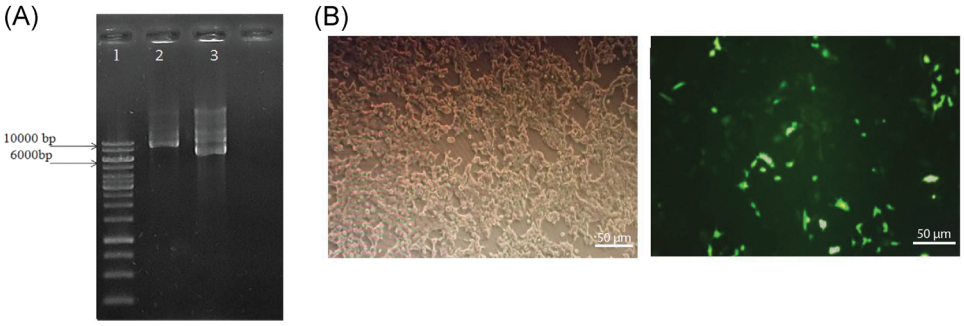

FIGURE 4 Production of recombinant retroviruses. (A) Evaluation of the extracted plasmids, on 1% gel, (1) 1 kb DNA ladder and (2) pAX with 10073 bp (3) pMD2 with 5824 bp. (B) Transfected HEK293T cells shows the expression of Green fluorescent protein (GFP) gene carried by the transfer vector (18 h after transfection). The image on the left shows the morphology and density of the HEK 293 cells under light microscope. Images were taken with a magnification of ×100. Scale bar = 50 µm

3.7 | Selection of higher producer clones

To provide monoclonal cells population with stable gene expression, limiting dilutions was used as described above.

From a 96 well plate, four separate clones were selected on the basise of GFP expression and moved to a 12-well plate for expansion (Figure 6).

3.8 | Assays for recombinant protein expression

Following clonal expansion, the real time PCR, ELISA, and Western blot tests were performed to screen and select the most appropriate clone with high expression of CD137-Fc protein.

3.8.1 | Enzyme-linked immunosorbent assay

Released CD137-Fc from the transduced CHO-K1 cell line was detectable in the cells culture supernatant by ELISA. Supernatants of each clone (1–4) were added to the anti-His Tag antibody coated wells. Following addition of HRP conjugated anti-IgG as detection antibody, the ELISA assay showed the binding of anti IgG to our fusion protein (CD137-Fc) (Figure 7A). There was no significant difference regarding the protein expression, between the selected clones except for the number 3, which was low producer.

3.8.2 | Result of real time PCR

Real time PCR was also performed to examine the mRNA expression by the isolated clones. The primary heterogeneous cell population was considered as control. As shown in Figure 7B, THE clone 4 showed the highest expression of mRNA compared to the others, which was consistent with the results of the ELISA and Western blot assay. (Figure 7B)

3.8.3 | SDS–PAGE and Western blot assay

SDS–PAGE electrophoresis showed the isolation of the CD137-Fc 70-kDa protein after affinity chromatography as demonstrated in Figure 7C. Following immunoblotting and Western blot analysis using anti-Human Fc and anti-His Tag antibodies in parallel, the resulted bands with the use of both antibodies demonstrate that CD137-Fc had a higher expression rate in clones 1 and 4 compared with others as shown in Figure 7D. Clone 1 was eventually selected for expansion based on the Western blot results.

3.9 | Assays for recombinant protein application

Preliminary in vitro tests were performed to confirm the efficacy of this fusion protein (CD137-Fc) as an immune system modulator.

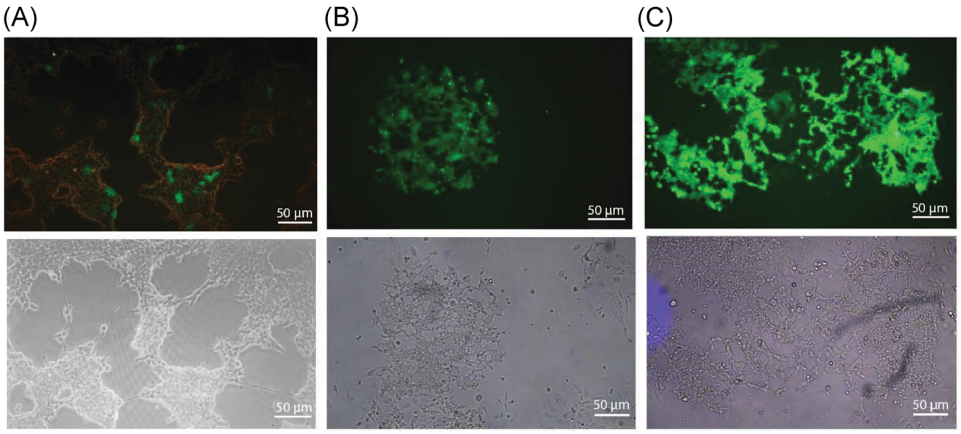

FIGURE 5 Confirmation of lentiviral transduction. Fluorescent microscopic images of the CHO-K1 cells, 24, 48 and 72 h following viral particle transduction. The image in the bottom row shows the morphology and density of the cells. Original magnification ×10. Scale bar = 50 µm

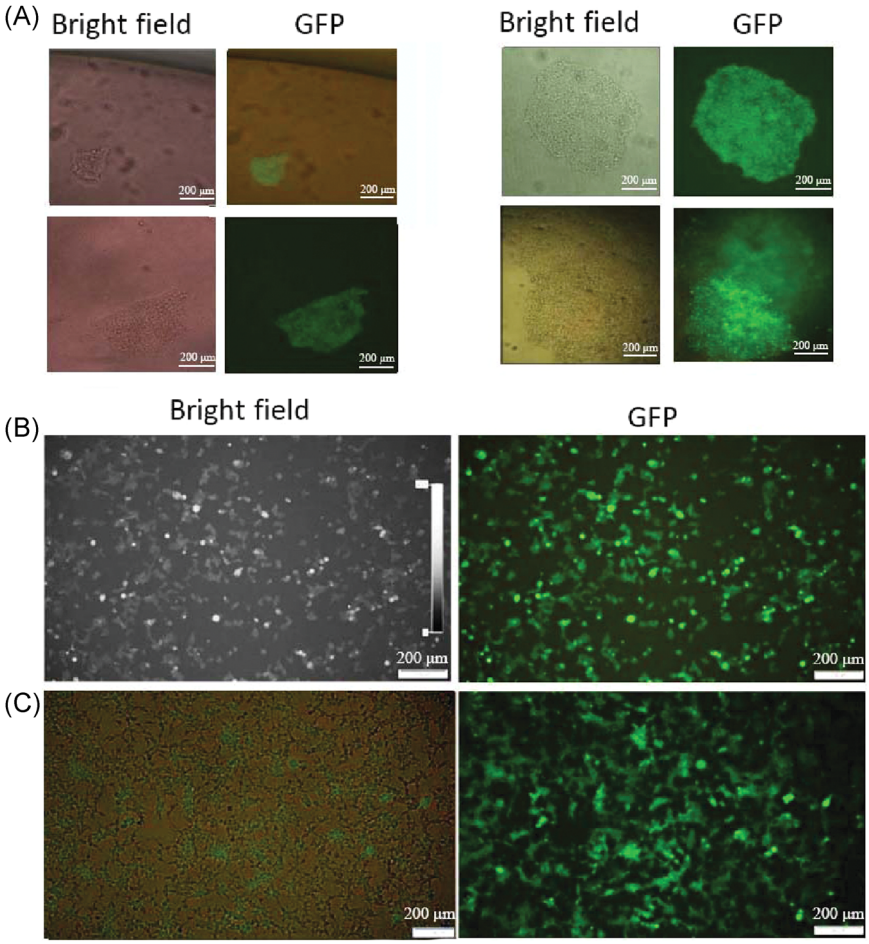

FIGURE 6 Preparation of homogeneous transduced cell population. To obtain a population of cells with stable gene expression the cells were seeded in a 96 well plate at an average density of 0.5 cells per well. The higher intensity of green florescent signal confirms the higher production of recombinant protein. The figure shows the fluorescence expression of one selected clone during subculturing in (A) 96 well plate. (B) 12 well plate. (C) T25 cell culture flask. Images are shown with a ×10 lens. Scale bar = 200 μm

3.9.1 | The CD137L-DC endocytosis capability was more than common DC

Dendritic cells express receptors on their surface that mediate the active uptake of dextran, the receptor-mediated endocytosis capacity of these cells can be shown in this way. The CD137L-DC endocytosis rate was significantly higher than common-DC. As shown in Figure 8A,B, immature CD137L-DCs exhibited capacity to incorporate FITC-dextran up to 82%, which in the mature form reaches to 62.6%. However, the capacity of dextran endocytosis in immature common DCs was 69.7%, which changed to 56.3% in the mature form. ($p < 0.01$).

3.9.2 | The IL-12P70 production of CD137L-dendritic cells upon their activation was higher than the common dendritic cells

The level of inflammatory cytokine secretion by dendritic cells is an indicator of its ability to activate T cell response. CD137L-dendritic cells released IL12p70 cytokine four times more than common DC under the influence of CD40-CD40L from T cells. CD137L-DC and common DC were cocultured with allogeneic T cells at 1:3 ratio of DCs:T cell. The obtained results is shown in Figure 8C.

3.9.3 | IL-6 and IL-8 decreased significantly in the samples exposed to CD137-Fc protein

CD137-Fc was able to reduce the secretion of IL-6 and IL-8 cytokines by almost half compared to the control in the leukocyte-rich plasma samples from hospitalized COVID-19 patients, indicating a decrease in inflammation and control of cytokine release syndrome. The results are shown in Table 3 and Figure 8D. The patient samples without CD137-Fc protein exposure was considered as control sample.

4 | DISCUSSION

CD137 is a member of the tumor necrosis factor receptor superfamily, which is expressed on both innate and adaptive immune cells.[6] In addition to its first known

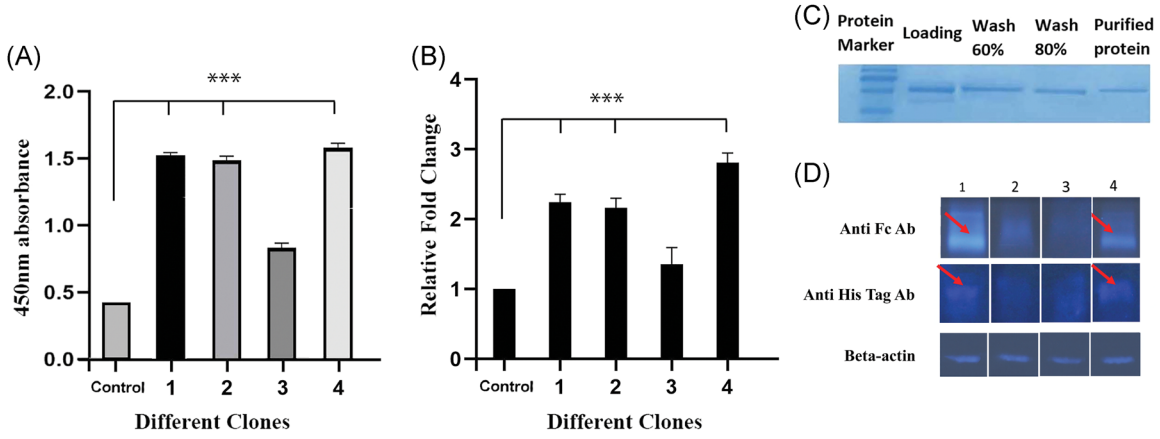

FIGURE 7 Evaluation of recombinant protein expression. Following the preparation of a homogeneous cell population, the resulted clones were examined for expression of the recombinant CD137-Fc protein by ELISA, real-time PCR, and Western blot analysis. (A) The expression of this fusion protein was measured in four isolated clones using the ELISA method. The primary heterogeneous population was considered as control. The results are expressed as optical density (OD) at 450 nm. ELISA assay was done as triplicate and the results are shown the mean ± *SEM* of three separated experiment ($n = 3$). One-way ANOVA was used to test for differences between groups. (***stands for $p < 0.001$). (B) The real-time PCR assay was used to evaluate the expression of recombinant gene in four isolated clones. The heterogeneous primary cell population was considered as control. Clone 4 was determined to have the highest expression of mRNA compared to the others, the experiments were performed in triplicate and the results are shown as mean ± *SEM* ($n = 3$). One-way ANOVA was used to test for differences between groups. (***stands for $p < 0.001$). (C) The SDS-PAGE results before immunoblotting shows the purification of a single band by affinity chromatography in the last washing. (D) The expression of the expected 70 kDa recombinant protein was detected in Western blot analysis without any degradation by anti-human IgG and anti-HisTag antibodies in separate tests indicating the existence of recombinant protein. CD137-Fc had a higher expression rate in clones 1 and 4 compared with others. ANOVA, analysis of variance; ELISA, enzyme-linked immunosorbent assay; IgG, immunoglobulin G; PCR, polymerase chsin reaction; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis

role in stimulating the T-lymphocytes, CD137-CD137L interaction is also able to activate the cells that express CD137L through CD137L reverse signaling which leads to multiple outcomes, including inflammatory cytokines secretion.[8,14,20]

Various approaches to the production of recombinant protein have been proposed, though they have some drawbacks. In protein synthesis by plasmid transfection of animal cell line, the probability of entry into the genome is usually very low, which is why the expression is almost temporary and limited to 2–3 days. Transduction method was introduced to solve the instability of protein synthesis using the lentiviral vectors. Moreover, production output significantly improved by this method.[21,22] We believe that we have developed an efficient method to stable production of the recombinant CD137-Fc protein.

In the following paragraphs, we will address the possible applications of recombinant CD137-Fc protein in the clinical setting.

CD137-Fc can be used as a costimulatory molecule in tumor immunotherapy, including the induction of inflammatory dendritic cells (CD137L-DC)[23] and the construction of chimeric receptor systems.[7] Due to its role in increasing stimulation of T cells, CD137-Fc can produce a potent response to kill tumor cells and virus-infected cells.[24]

The produced CD137-Fc fusion protein not only behaves like the membrane CD137 and activates the immune response when it is fixed via the Fcγ receptor on the T cell surface,[25,26] but can also compete with mCD137 in the released (soluble) form for interaction with its ligand and act as an immune regulator, just like the sCD137.[27,28]

Activated T cells may express FcγRIIIa, and engagement of these receptors contributes to effector T cell development. Briefly, a hetero dimer is formed from the Fc receptor common γ-chain and the ζ-chain of T-cell receptor complex, causing the synergistic function of the receptors.[26]

Consistent with our finding, recent research show that CD137-Fc can be used as a novel immune modulator for the treatment of tumors, autoimmune diseases and viral infections.[2,16,29]

In COVID-19 sever cases, the presence of an acute and damaging immune reaction sustained by cytokines leading to alveolar infiltration by macrophages and monocytes was shown.[30] Based on preliminary research, targeting the cellular base of cytokine storm induced by COVID-19 using CD137-Fc could be a

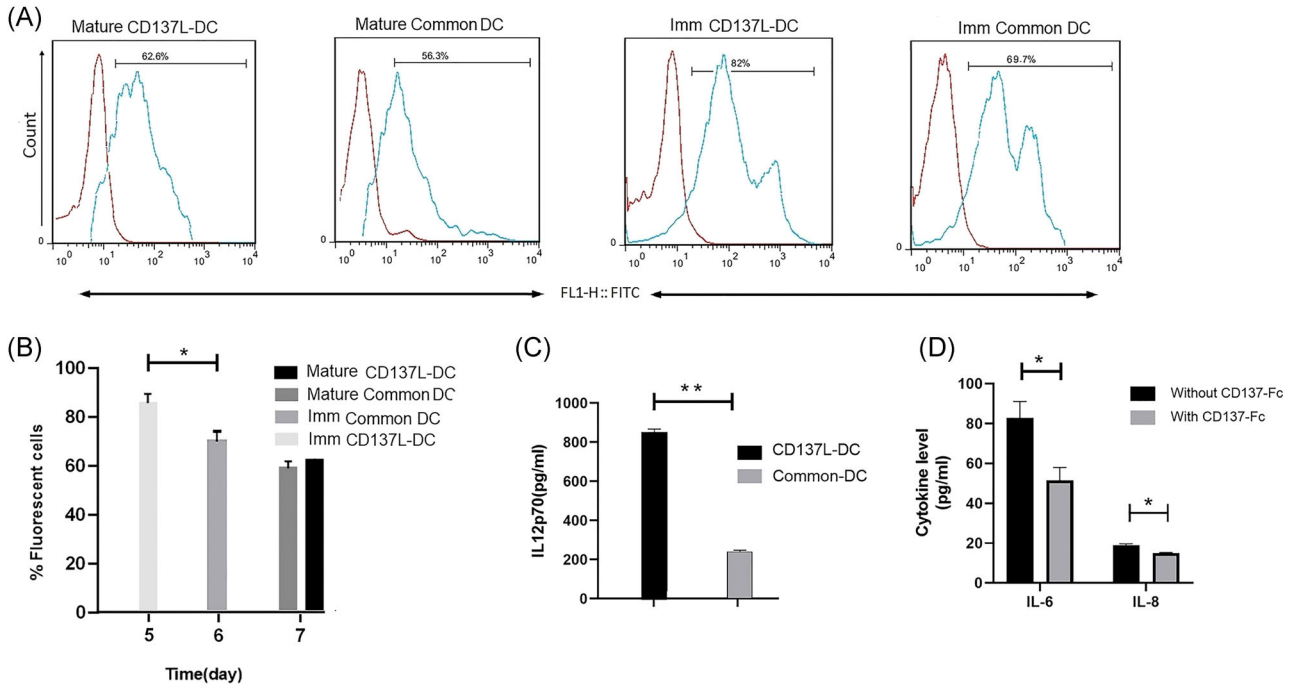

FIGURE 8 Evaluation of the applications of CD137-Fc fusion protein. Blood monocytes were cultured on plates coated with 10 μg/ml CD137-Fc protein or in the present of 100 ng/ml GM-CSF and 20 ng/ml IL-4, then analyzed using the flow cytometry. (A) Endocytosis: FITC-conjugated dextran were added to the cells for 1h before flow cytometry analysis. The histograms show a representative of three different experiments. The blue histogram shifts to the right indicates the FITC-dextran uptake by the cells. The red histograms show the control cells. (B) A quantitative comparison was made between immature and mature dendritic cells in both groups based on the Mean ± *SEM* ($n = 3$) of flow cytometry data obtained from separate tests. (**stands for $p < .01$). (C) CD137L-DC and common DC were co-cultured with T cells at ratio of 1:3 DCs: T cell for 12 h. CD137L-DC released IL12p70 cytokine four times more than common DC. The results are Mean ± *SEM* of three different experiments. (**stands for $p < 0.01$). (D) COVID 19 patients' samples were cultivated with and without CD137-Fc protein. The changes of the IL-6 and IL-8 levels in cultured cells supernatants were measured using the ELISA method. The results show the mean ± *SEM* of five different experiments. COVID-19, coronavirus disease 2019; ELISA, enzyme-linked immunosorbent assay; FITC, Fluorescein isothiocyanate; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin

TABLE 3 Cytokine levels in culture supernatant of COVID-19 patients' leukocytes in the presence and absence of CD137-Fc protein

	IL-6 (pg/ml)		IL-8 (pg/ml)	
Study group	**Without CD137-Fc**	**With CD137-Fc**	**Without CD137-Fc**	**With CD137-Fc**
No.	5	5	5	5
Mean ± *SEM*	82.84 ± 8.225	51.42 ± 6.512	19.04 ± 0.512	15.01 ± 0.274
p Value	<0.05		<0.05	

Note: Sample: leukocyte-rich plasma of COVID-19 patients. The statistical analysis was performed using the Mann–Whitney test.

Abbreviations: COVID-19, coronavirus disease 2019; IL. interleukin.

possible therapeutic option, along with supportive care strategies, to improve the outcomes of the disease.

The soluble CD137 (sCD137) is released as a natural regulator at the maximum excitation intensity of the T-cell. Similar to sCD137, recombinant CD137-Fc competes with mCD137 to bind to the ligand. This prevents further T-cell stimulation to stop damage to the self-tissue.[3,12]

On the other hand, the recombinant CD137-Fc induces cross-linking and transduction of signals by CD137L. It triggers downstream reactions that activate ligand-expressing cells and secretes inflammatory cytokines. It can be considered as a treatment for some immunodeficiency or cancerous diseases.[8,31,32]

CD137–CD137L interactions may have a significant effect on the maintenance of CD8 + T-cell responses in

viral infections.[4,33,34] It can therefore play an important role in generating a memory response in COVID-19 patients as well.

The use of our product as an antiviral agent is important because of the recent coronavirus pandemic state.

5 | CONCLUSION

Recombinant CD137-Fc, whether soluble or surface-coated via Fc, will have several effects on the modulation of the immune system, such as enhancing both antiviral and antitumor responses, while alleviating some autoimmune conditions. It may reduce cytokine-released syndrome and lung tissue inflammation due to the coronavirus infection. Its use, along with other antiviral therapy, should be considered as a potential approach to the treatment of patients with a COVID-19 virus infection.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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SUPPORTING INFORMATION

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