Construction and Expression of Transmembrane and Secretory Recombinant Human CD4 Extracellular Domain in HEK293F Cell

ABSTRACT

Cluster of differentiation 4 (CD4) is a common receptor and signal transduction molecule expressed on CD4+ cell membrane. CD4 molecules involve in the identification of major histocompatibility complex II molecules, which plays an important role in the development and differentiation of T lymphocyte subsets and CD4+ cell antigen recognition. The extracellular domain of CD4 protein contains a lot of epitopes, which is more potent than that of antigen peptide molecules. In this study, we engineered and constructed an extracellular domain of CD4 protein (CD4_{ECD}) and an CD4_{ECD}-Fc fusion protein. The recombinant vectors were transfected into HEK293F cells, and then expressed and purified. Functional assay showed that the CD4_{ECD} was successfully expressed on the cell membrane, and the CD4_{ECD}-Fc fusion was obtained in cell supernatant. Therefore, we successfully expressed transmembrane and secretory CD4_{ECD} proteins.

KEYWORDS cluster of differentiation, major histocompatibility complex (MHC), T lymphocyte.

INTRODUCTION

Since 1981, the United States found and reported that patients with acquired immunodeficiency syndrome (AIDS), human immunodeficiency virus (HIV) has rapid spread in the global, there have been 22 million people died of AIDS, now AIDS has become the fourth largest cause of human death\(^1\). As the pathogen caused AIDS, over the years scientists have been working hard to study the pathogenesis of HIV, looking for effective antiretroviral therapy targets, designing HIV vaccines and diagnostic methods to achieve the ultimate goal of controlling AIDS. But at present, AIDS antiviral therapy and specific immune prophylaxis are far from being resolved. Studies found that the binding of HIV to the cell surface of this process relies on the binding of the viral surface protein gp120 to the specific host cell surface receptor, the most important of which is the CD4 receptor.

Cluster of differentiation 4 (CD4) is a co-receptor and signal transduction molecule, which is mainly expressed on the CD4+ cell membrane. The CD4 molecule is a single chain transmembrane glycoprotein with a molecular weight of about 55 kDa and belongs to the immunoglobulin superfamily. Its extracellular molecular weight is about 42 kDa. CD4 molecule participates in the recognition of major histocompatibility complex (MHC) class II molecules, and plays an important role in the development and differentiation of T lymphocyte subsets, and in the process during CD4+ cell antigen recognition\(^2\). However, when the normal immune function of CD4 molecules dysfunction or damage, it may lead to disease. Thus, the CD4 molecule becomes a target for many diseases, such as transplant rejection, rheumatoid arthritis, Crohn’s disease, multiple sclerosis, cancer, Sjogren syndrome, psoriasis, asthma, atopic dermatitis, autoimmune diabetes and AIDS treatment and HIV infection and so on\(^3\).

CD4 molecules not only play an important role in the process of the immune response, but also be the receptor of some virus invading the body’s immune system. The mechanism of HIV infection in the human body has been clear. Human CD4 molecule as the first receptor of HIV membrane...
gp120 is a key step in its invasion of CD4+ T cells, mononuclear macrophages and so on. After binding to gp120, gp120 undergoes homogenization and exposes the second receptor CCR5 and CXCR4 binding sites, which in turn activate the HIV outer membrane gp41 and insert into the cell membrane, and finally completes the fusion of the viral envelope with the host cell membrane. Li et al. also demonstrated that the interaction of CD4+ T cells with B cells is the key to pathogenesis in mouse AIDS models. Thus, monoclonal antibodies against CD4 molecules can theoretically prevent and treat AIDS, and in fact in clinical trials also showed a certain potential.

Tumor vaccines are designed to enhance the immune system’s ability to recognize tumors, improve immune microenvironment, and stimulate specific cellular immunity by introducing tumor antigens into patients. Cancer vaccines currently under development and entering clinical trials include the following categories: Tumor cell vaccines, divided into all tumor cell vaccines and genetically modified tumor cell vaccines; Tumor antigen vaccine, divided into tumor-associated antigen (TAA) and tumor-specific antigen (TSA) vaccine. Tumor antigens also include multiple levels, such as intact protein molecules, antigenic peptides and purified DNA; Tumor DNA vaccine, in fact, is a kind of antigen vaccine, is divided into naked DNA, plasmid DNA and viral vector DNA vaccine; DC vaccine is TAA directly into the DC, and successfully expressed secreted and transmembrane proteins, a reducing SDS-PAGE and region of the protein, ECD was confirmed by colony PCR and sequencing. The gene of CD4 and IgG Fc fragment was amplified by PCR using cCD4ECD-F-2 (ATAAAAGGTACCATGAACCGGGGA GTCCCTTT) and cCD4ECD-R-2 (GTGCCAGCTTGGGTCAGCTGGCAC CCGGGTG), cFc F (CACC CGGCCAGAGGC CAAAGCCTGCGAC) and cFc R (ATAAAAGGTACCATGAC TCTTGCCGCGGCG). The recombinant vector was transformed to DH5α and the sequence of plasmid pCMV-CD4ECD-Fc was confirmed by colony PCR and sequencing. Fig 1.

**Bacterial strains, media and culture conditions**

The E. coli strain DH5α was stored in our laboratory, and used as the host strain for cloning and maintenance of plasmids throughout the experiments. The pDisplay and pCMV (CHENDU Transvector Biotechnology Co. Ltd) were used as expression vectors for protein expression. The HEK293F cell was maintained at 37°C in 5% CO2 and 95% air.

**Protein expression**

The vectors (pDisplay-CD4ECD/pCMV-CD4ECD-Fc) were amplified in E. coli cells and DNA was purified using Endo Free Plasmid Maxi Kit (Qiagen). HEK293 cells in the exponential growth phase were grown in Gibco FreeStyle 293 Expression Medium (Invitrogen) until they reached a cell density of 1 × 10^6 cells/ml. The cells were transiently transfected using FreeStyle MAX Reagent (Invitrogen) according to the manufacturer’s instructions. Briefly, 120 μg DNA diluted in Gibco OptiPro SFM (Invitrogen) were gently mixed with 120 μl FreeStyle MAX Reagent diluted in OptiPro SFM and incubated for 10 min. The mixture was added drop-wise to a flask containing 150 ml HEK293F cells. The transfected cells were allowed to grow in suspension for 6 days at 37°C in a humidified atmosphere of 5% CO2 on an orbital shaker platform rotating at 135 rpm. The blank control group without transfection was set.

**SDS-PAGE and western blot analysis**

To evaluate the protein expression of the transmembrane and secretory CD4ECD proteins, a reducing SDS-PAGE and western blotting were performed on the samples. The collected cells were digested with 1% SDS at 100°C for 10 min, and the supernatant was used to detect the total protein concentration by BCA to adjust the same protein concentration in the samples.

For western blotting, the proteins were transferred from the second gels to a nitrocellulose membrane (GE Healthcare, UK), where residual protein-binding sites were subsequently blocked with 5% BSA. In order to detect the human CD4ECD region of the protein, the membrane was incubated with anti-human CD4 HRP antibody (Sigma, Germany) at 4°C overnight, and...
goat anti-mouse IgG-HRP antibody was made as secondary antibody. Finally, the bands were revealed by 3, 30-Diaminobenzidine (DAB) solution (Sigma, Germany).

**Flow fluorescence analysis**

After 48 h, 5 × 10^6 cells transfected by pDisplay-CD4\text{ECD} were washed with 1 × PBS, fixed with 5% paraformaldehyde. Anti-human CD4 mouse antibody was used as primary antibody, goat anti-mouse IgG-FITC antibody was made as secondary antibody. The cells of blank control group were set the fluorescence domain values. Then the expression of CD4\text{ECD} on HEK293F cell membrane was detected by Guava micro-capillary cell analyzer.

**Spectral laser scanning confocal microscopy fluorescence detection**

After 48 h, 1 × 10^5 transfected cells were washed with 1 × PBS, fixed with 5% paraformaldehyde, increased the permeability with triton × 100, and blocked with 5% BSA. Anti-HA mouse antibody was used as primary antibody, goat anti-mouse IgG-FITC antibody was made as secondary antibody. Moreover, anti-human CD4 antibody (with phycoerythrin PE) was directly incubated. Then, the intracellular DNA was stained with DNA dye DAPI, and the treated cells were examined by spectroscopic laser scanning confocal microscopy.

**Purification and identification of CD4\text{ECD}-Fc**

The cell supernatant with CD4\text{ECD}-Fc was filtered with 0.45 filter and purified by protein A affinity chromatography column. The mixture of column was equilibrated with 20 mM PB (sodium dihydrogen phosphate). Then the targeted protein was eluted with 100 mM glycine, whose pH was adjusted to 7.2 with Tris, the purity was analyzed by SDS-PAGE and HPLC.

### Results

**Construction of pDisplay-CD4\text{ECD} and pCMV-CD4\text{ECD-Fc}**

The gene fragment CD4\text{ECD} and Fc were amplified by PCR, and detected by agarose gel electrophoresis. As shown in Fig. 2A, the amplified CD4\text{ECD} fragment was about 1100 bp, the amplified Fc fragment was about 700 bp, which was in accordance with the predicted size of CD4\text{ECD} and Fc gene. then, overlapping PCR was used to obtain the CD4\text{ECD}-Fc, as shown in Fig. 2B, the amplified fragment was about 1800 bp, which was in accordance with the predicted size of CD4\text{ECD}-Fc. Colony PCR as shown in Fig. 2C and sequencing the positive colonies verified that the results was 100% matched with CD4\text{ECD} and CD4\text{ECD-Fc} gene sequence, indicating that pDisplay-CD4\text{ECD} and pCMV-CD4\text{ECD-Fc} were successfully constructed.

**Western blotting test of CD4\text{ECD} and CD4\text{ECD-Fc}**

The supernatants of cell transfected by pDisplay-CD4\text{ECD} and pCMV-CD4\text{ECD-Fc} were analyzed by Western Blotting. The results of CD4\text{ECD} were shown in Fig. 3A. There were only one targeted band with a size of 42 kDa of CD4\text{ECD}. Further measurement of the internal β-actin were carried out with a size of 43 kDa in line with its expected size. Fig. 3B showed the result of CD4\text{ECD-Fc} that only one targeted band with a size of 42 kDa consistent with predicted protein size. The above results confirmed the successful expressing of CD4\text{ECD} and CD4\text{ECD-Fc}.

**Flow fluorescence analysis of expression of CD4\text{ECD} on HEK293F cell membrane**

The cells in the blank control group and experimental groups were analyzed by flow cytometry. As shown in Fig. 4, the three experimental groups (Fig. 4B–D) showed positive results compared with the blank
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Fig. 2 Construction of pDisplay-CD4<sub>ECD</sub> and pCMV-CD4<sub>ECD</sub>-Fc. A: Amplification of CD4<sub>ECD</sub> and Fc, B: Overlapping PCR for CD4<sub>ECD</sub>-Fc, C: Colony PCR of pDisplay-CD4<sub>ECD</sub> and pCMV-CD4<sub>ECD</sub>-Fc.

Fig. 3 Expression of CD4<sub>ECD</sub> and CD4<sub>ECD</sub>-Fc. C: WB test of CD4<sub>ECD</sub> expression, D: WB analysis of CD4<sub>ECD</sub>-Fc expression.

Fig. 4 Flow fluorescence analysis of expression of CD4<sub>ECD</sub> on cell membrane. A: Blank control group. B, C, D: Three parallel experimental group.

Identification and purification of CD4<sub>ECD</sub>-Fc

After 150 h of cell transfection, the supernatant was collected to purify by protein A affinity column. 20 mM PB (sodium dihydrogen phosphate) was used to equilibrate, and 100 mM glycine was utilized to elute target protein whose pH was adjusted to 7.2 with Tris. Fig. 6A is the purification chromatography chart of CD4<sub>ECD</sub>-Fc. The red arrow refers to the elution peak of the targeted protein. The purified protein was subjected to SDS-PAGE electrophoresis. As shown in Fig. 6B two lanes of SDS-PAGE were three parallel-purified samples, and the amount of protein was 5 µg. There was only one targeted band in the lanes, the size of 70 kDa, consistent with the theoretical value and results of western blotting test. Moreover, it showed that the purified CD4<sub>ECD</sub>-Fc protein was with high purity. The purity of CD4<sub>ECD</sub>-Fc protein was 97.50% verified by HPLC (Fig. 6C).

DISCUSSION

Unlike previous surgical excision, chemotherapy, radiotherapy and gene targeted therapy, cancer immunotherapy is aimed at a patient’s own immune system to fight cancer. In recent years, monoclonal antibodies against immune checkpoints have created a revolution in clinical oncology, which is an important milestone on the journey of cancer therapy. The success of checkpoint blockades is slowly driving researchers away from the genetic view of cancer to immune-based approaches. At present, with control group (Fig. 4A). The positive rate respectively were 40.93%, 41.52%, 41.09%, indicating CD4<sub>ECD</sub> was successfully expressed in the cell membrane.

Spectral laser scanning confocal microscopy fluorescence detection for expression of CD4<sub>ECD</sub> on HEK293F cell membrane

The cells in Fig. 5A–D were with anti-HA mouse antibody as primary antibody, goat anti-mouse IgG-FITC antibody as secondary antibody. The positive results will show the cell surface with green fluorescence. Fig. 5A is blank control group, the cell surface without any fluorescence imagination. Fig. 5B–D are three parallel experimental groups, the cell surface with green fluorescence. It indicates that CD4<sub>ECD</sub> was successfully expressed on the cell membrane.

The cells in Fig. 5E–H were directly incubated with anti-human CD4 antibody. The positive results will show the cell surface with red fluorescence. Fig. 5E is blank control group, the cell surface without any fluorescence imagination. Fig. 5F–H are three parallel experimental groups, the cell surface with red fluorescence. It indicates that CD4<sub>ECD</sub> was successfully expressed on the cell membrane again.

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DISCUSSION

Unlike previous surgical excision, chemotherapy, radiotherapy and gene targeted therapy, cancer immunotherapy is aimed at a patient’s own immune system to fight cancer. In recent years, monoclonal antibodies against immune checkpoints have created a revolution in clinical oncology, which is an important milestone on the journey of cancer therapy. The success of checkpoint blockades is slowly driving researchers away from the genetic view of cancer to immune-based approaches. At present, with
Construction and expression of transmembrane and secretory recombinant human CD4.

**Fig. 5** Confocal microscopy detection for CD4 expressed on the cell membrane. A: Blank control, B-D: Three parallel experimental groups, E: Blank control, F-H: Three parallel experimental groups.

**Fig. 6** Identification and purification of CD4-Fc protein. A: Chromatography of CD4-Fc purified by Protein A, B: SDS-PAGE and WB of CD4-Fc, C: HPLC for CD4-Fc.
the rapid development and cross-infiltration of pharmacology, molecular biology, tumor immunology and other related disciplines. As well as the development of tumor-associated antigens, vectors and adjuvants, various forms of tumor vaccines have been developed and entered into clinical trials, all these include tumor cell vaccines, tumor antigen vaccines, DNA vaccines, DC vaccines, and bacterial vaccines, which have contributed to tumor immunotherapy.

In this study, the transmembrane and secretory CD4 ECD were made, which was the first step of obtaining the high biological activity anti-CD4 antibody through immune response. pDisplay and pCMV were used as vectors for the expression of transmembrane and secretory CD4 ECD, respectively. There is a PDGFR transmembrane domain in pDisplay. The PCR products of CD4 ECD and CD4 ECD–Fc were, respectively, cloned to pDisplay and pCMV to form the recombinant plasmids of pDisplay–CD4 ECD and pCMV–CD4 ECD–Fc. At present, researchers have expressed CD4 protein in E. coli. However, the protein expressed by E. coli is present as an inclusion body, which needs to be purified and renatured to become a bioactive soluble protein. Therefore, in this study, we use HEK293F eukaryotic expression system. The HEK293F cells transfected by pDisplay–CD4 ECD were detected by Western Blotting, flow cytometry and confocal microscopy, and the results showed that CD4 ECD was successfully expressed on the membrane. The supernatant of HEK293F cells transfected by pCMV–CD4 ECD–Fc was confirmed by Western Blotting, using protein A affinity chromatography to purify the protein. Protein purity was verified by SDS–PAGE and HPLC, so the CD4 ECD–Fc fusion with high purity of 98.10% was obtained.

It needs to solve the problem of immunogenicity when making CD4 ECD as the tumor protein vaccine. CD4 ECD has weak immunogenicity, which must be used in combination with immune adjuvant, so to be uptaken and processed by antigen-presenting cells. In recent years, it has been found that the complex of HSPs in tumor cells and tumor antigenic proteins was used to immunize homologous animals to induce specific anti-tumor cell immunity. Therefore, the CD4 extracellular domain protein could be a promising tumor protein vaccine. The transmembrane and secretory CD4 ECD proteins expressed in this study were prepared for the preparation of the CD4 extracellular vaccine. It is expected that through vaccine immunization the anti-CD4 antibody with better properties of physical and chemical and higher biological activity will be obtained, it is not only conducive to the further development of immune biology, diagnosis and treatment research of CD4 high expressed positive tumors, but also a good material to study the structure and function of human CD4 molecular.

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