Expression and Purification of the Recombinant Human CTLA-4 Extracellular Domain and Screening and Functional Identification of its Nanobody

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Abstract

CTLA-4 is a highly expressed negative immune checkpoint in many solid tumors. Nanoantibody has the characteristics of high thermal stability, affinity, permeability, high expression and easy purification, so it is the first choice to develop CTLA-4 inhibitors. In order to obtain human CTLA-4 nanoantibody, we constructed CTLA-4 ECD recombinant plasmid, and screened NbCTLA-4 ECD as F2, E3, G4 by phage display technology, and transferred pMECS-NbCTLA-4 ECD into E.coli WK6. The results showed that the yield of G4 was the highest, reaching 5.84 mg / L, and the specific binding ability with CTLA-4 ECD was stronger than that with other six control antigens. NbCTLA-4 ECD had obvious advantages compared with the positive control CTLA-4 mAb at 60 ℃. The results of flow cytometry showed that the three nanoantibodies could recognize CTLA-4 expressed on cell membrane surface, and G4 had the highest binding activity.

Keywords

Cytotoxic T Lymphocyte-Associated Antigen 4 (CTLA-4); Nanobody; Phage Display Technology.

1. Introduction

Immune checkpoint, also known as costimulatory molecules, mainly regulates the immune response mediated by T cells in the specific immune process of the body. It can be divided into two kinds of costimulatory molecules: activation and inhibition. Obviously, one part of them is to promote the activation of T cells and make them play an immune effect; the other part is to inhibit the activation or over activation of T cells [1, 2]. Clinical studies have shown that inhibitory costimulatory molecules mainly include cytotoxic T lymphocyte associated antigen-4 (CTLA-4), programmed death molecule-1 (PD-1), ligand-1 of programmed death molecule-1 (PD-L1), T cell membrane protein-3 (Tim-3), lymphocyte activating gene-3 (LAG-3), etc.[3] CTLA-4, also known as CD152, is a transmembrane receptor on T cells and a member of the CD28 family. CTLA-4 is also one of the most widely studied inhibitory co receptors. Studies have shown that CTLA-4 has a strong inhibitory effect on the phenotype of CTLA-4 deficient mice. In addition, the binding of CTLA-4 with ligands may have a negative effect on cell cycle regulation[4, 5]. In clinical trials, anti CTLA-4 antibodies have shown efficacy in the treatment of prostate cancer, malignant melanoma and (small cell and non-small cell) lung cancer[6, 7]. Recently, there is a probiotic bacteria system for the controlled production and intratumoral release of nanobodies targeting programmed cell death-ligand 1 (PD-L1) and cytotoxic T lymphocyte-associated protein-4 (CTLA-4) using a stabilized lysing release[8]. Due to their small molecular weight and simple structure, nanoantibodies are only transcribed from heavy chain variable region genes. Some results show that the production of nanoantibodies can be increased to 500 mg/L through yeast reactor[9, 10]. Nanoantibodies have the advantages of high stability, high specificity and easy purification, so they are very suitable for biological probes[11]. Studies have

shown that there are nano antibodies against the prostate, which can be used for early detection of prostate cancer[12]. Nanoantibodies can also be combined with optical imaging technology and ultrasonic imaging technology, and can be used for quantitative diagnosis of clinical diseases and early auxiliary diagnosis of cancer [13-15]. It has been reported that researchers have developed nano antibodies against scorpion and snake venoms, and can effectively neutralize the toxins[16-19]. BMS has developed a monoclonal antibody targeting CTLA-4, yervoy (ipilimumab) which has been approved by FDA for the treatment of advanced metastatic melanoma. The company also began to study the clinical trials related to the treatment of cancer with antibodies combined with drugs [20-22]. The purpose of this study is to screen the nano antibody binding to CTLA-4 through the large capacity phage nanoantibody library, so as to provide materials and basis for antibody drugs in tumor immunotherapy.

2. Materials and methods

2.1 Material and reagesnts

pcDNA 3.4, E. coli DH5α, A375 cells were supplied by our laboratory. pCMV, HEK 293F kindly provided by Peking University. Prime STAR HS DNA Polymerase was purchased from Takara. 2× Taq PCR Master Mix was purchased from TIANGEN. Fast Digest Kpn I, Fast Digest Xho I, Fast Digest Nhe I, Fast Digest Hind III were purchased from Fermentas. Anti-CTLA-4 mAb was purchased from Abcam. Goat anti-mouse IgG mAb was purchased from abcam CST. RPMI 1640 Medium, DMEM Medium, Fetal Bovine Serum (FBS) were purchased from Gibco. CTLA-4, PD-1, PD-L1, CD4, CD8a, CD28, CD80 were purchased from Yiqiao Shenzhou company.

2.2 Instruments

AKTA primer (GE). SDS-PAGE electphoresis (Bio-Rad). Cell incubator (Eppendorf). Flow cytometry (BD). Elisa Plate Readers (Thermo). High-speed low-temperature centrifuge (Eppendorf). Metal bath (Hangzhou Ruicheng Instrument Co., Ltd.). Gel imaging system (Bio-Rad).

2.3 Construction of expression vector pCMV-CTLA-4 ECD and pcDNA3.4-CTLA-4 ECD

The CDS region of human CTLA-4 extracellular region was found from the Uniprot databases, and a signal peptide was added in front of its N-terminal, and a segment of his tag and a terminator were added behind the C-terminal. Then, referring to the polyclonal site information of PCMV vector, the CDS region of CTLA-4 was synthesized and constructed on pCMV vector (KpnIand XhoIwere selected) and used E. coli DH5α as host.

According to the base sequence of CTLA-4 extracellular segment and the restriction site information of pcDNA3.4, a pair of primers were designed to subclone the extracellular segment of CTLA-4 into pcDNA3.4 vector,the expression plasmid pcDNA3.4-CTLA-4 ECD was subcloned by PCR amplification, restriction enzyme digestion, ligation and transformation.

2.4 Expression, purification and identification of CTLA-4 ECD

When HEK 293F cells were cultured to logarithmic growth phase and the survival rate was more than 90%, pCMV-CTLA-4 ECD was transformed into cells by PEI according to the ratio of plasmid to PEI of 1:2. Cell viability and density were measured every day, and a small amount of cell supernatant samples were collected until the viability was lower than 50%. The collected samples were purified by Ni Sepharose TM excel column, and its labeled protein could be captured. 1×PBS (pH 7.4) was used as washing buffer and 80 mm imidazole elution buffer was contained. Then, the imidazole in eluent was removed by ultrafiltration tube. The CCF of HEK 293F cells and CCF of pCMV-CTLA-4 ECD transformed HEK 293F cells were analyzed by SDS-PAGE and Western blotting.

2.5 Quality identification of CTLA-4 ECD

The purity of the antigen screened by nanoantibody elution is not only higher than 90%, but also the biological activity of the antigen protein. The CTLA-4 ECD protein was coated by ELISA, 100ng / well, and then the diluted CTLA-4 mAb was added to combine with it. The absorbance value measured at 450nm wavelength was used as fitting curve to reflect the binding of antigen and antibody.

2.6 Auxiliary phage amplification

VCSM13 assisted phage was diluted gradiently, and 1 μL was taken to infect 50 μL TG1 bacterial solution in logarithmic growth phase. 3mL liquefied agar LB medium was added to each dilution, and then quickly and evenly poured into LB plate, and inverted into 37℃ incubator overnight. The number of plaques in the plate was calculated to obtain the titer of the auxiliary phage. A large number of VCSM13 helper phages were amplified by picking out a single plaque to infect TG1.

2.7 Elution and screening of NbCTLA-4 ECD by phage display technology

The purified CTLA-4 ECD protein was coated overnight on the enzyme plate, and the phage nanoantibody library with the capacity of 6.68 × 1011 CFU / total was used. After the first round of amplification and three rounds of elution, the number of times of elution in the first round was 5, and the number of transformants binding to CTLA-4 ECD was calculated size.

After the last round of washing, 96 single colonies were randomly selected from the plate with appropriate dilution, inoculated into 96 well deep well plate, placed in a shaking table at 28 ℃, 200 rpm, induced overnight by IPTG to express nano antibody. Because of the expression of nanoantibodies in periplasmic space, osmotic pressure difference method was used to extract periplasmic space proteins. The results showed that the phage had high affinity for CTLA-4 ECD when the absorbance value of OD450nm was more than twice that of the blank control pore. By ELISA, 18 positive transformants were screened out, and the plasmids were extracted and sent to the company for sequencing.

2.8 Analysis of physicochemical properties of NbCTLA-4 ECD

The physicochemical properties of NbCTLA-4 ECD amino acid sequence were analyzed by ExPASY website, including the prediction of molecular weight, isoelectric point and total average hydrophilicity.

2.9 Prokaryotic expression and purification of NbCTLA-4 ECD

Three strains of positive transformants were cultured in a large scale, and then the plasmid pMECS-NbCTLA-4 ECD was extracted. Three different plasmids were electroporated into the competent state of E.coli WK6. At least three positive clones of each plasmid were sequenced. The three WK6 strains were inoculated into TB / Amp medium for amplification. The expression of NbCTLA-4 ECD was induced by IPTG at 28 ℃ overnight.

There is a signal peptide pel B on PMECs phage vector. NbCTLA-4 ECD is finally expressed between the cell wall and cell membrane of WK6 strain (i.e. periplasmic space) under the guidance of signal peptide. Therefore, high osmotic pressure difference extraction method was used to obtain periplasmic space protein. Different elution peaks of imidazole were collected for sample preparation. SDS-PAGE electrophoresis was used to determine which elution concentration contained NbCTLA-4 ECD, and Western blot was used to test whether it was NbCTLA-4 ECD.

2.10 The specificity of NbCTLA-4 ECD was detected by elisa

PD-L1, PD-1, CD28 and CD80 were used as control antigens, while CD4 and CD8a were TCR. The binding specificity of three NbCTLA-4 ECD trains was determined by ELISA with the above six proteins as control antigens, PBS as blank control and mouseanti-CTLA-4antibody (CTLA-4 mAb) as positive control. The absorbance value reflects the binding specificity of NbCTLA-4 ECD and CTLA-4 ECD.

2.11 Determination of thermal stability of NbCTLA-4 ECD

CTLA-4 mAb and NbCTLA-4 ECD were placed at 25 ℃, 37 ℃, 60 ℃, 90 ℃ for 10 min, 30 min, 60 min, 120 min and 180 min respectively. The binding activity of different samples to CTLA-4 ECD was measured by ELISA, that is, whether NbCTLA-4 ECD still has the ability to bind to CTLA-4 ECD after different degrees of heat treatment.

2.12 Flow cytometry analysis of NbCTLA-4 ECD binding to CTLA-4 on A375 cell membrane

In order to study the biological activity of the nano antibody, the specific binding of the antibody to CTLA-4 molecule expressed on the cell surface was analyzed. Because of the high expression of CTLA-4 protein on the surface of A375 cells, A375 melanoma cells were used as experimental materials to analyze the binding of NbCTLA-4 ECD.

3. Results

3.1 Expression, purification and identification of extracellular domain of human CTLA-4

The synthesized pCMV-CTLA-4 ECD E.coliDH5 PCR was verified by colony PCR. The results of agarose gel electrophoresis were as follows. (Fig.1A), it was obvious that 12 mAbs had a clear band at the upper part of the 500bp, which was consistent with the 516bp of the synthetic fragment. The results showed that pCMV-CTLA-4 ECD recombinant plasmid was successfully synthesized.

The recombinant plasmid was sequenced by double enzyme digestion (NheI/Hind III), and two bands were obtained by agarose gel electrophoresis. As shown in (fig.2B), a 500bp was located on the top of 500bp, which was consistent with the CTLA-4 ECD gene 516bp. The other one was above 5000bp, which was consistent with the size 6011bp of carrier pcDNA3.4, indicating that the recombinant plasmid pcDNA3.4-CTLA-4 ECD was successfully constructed.

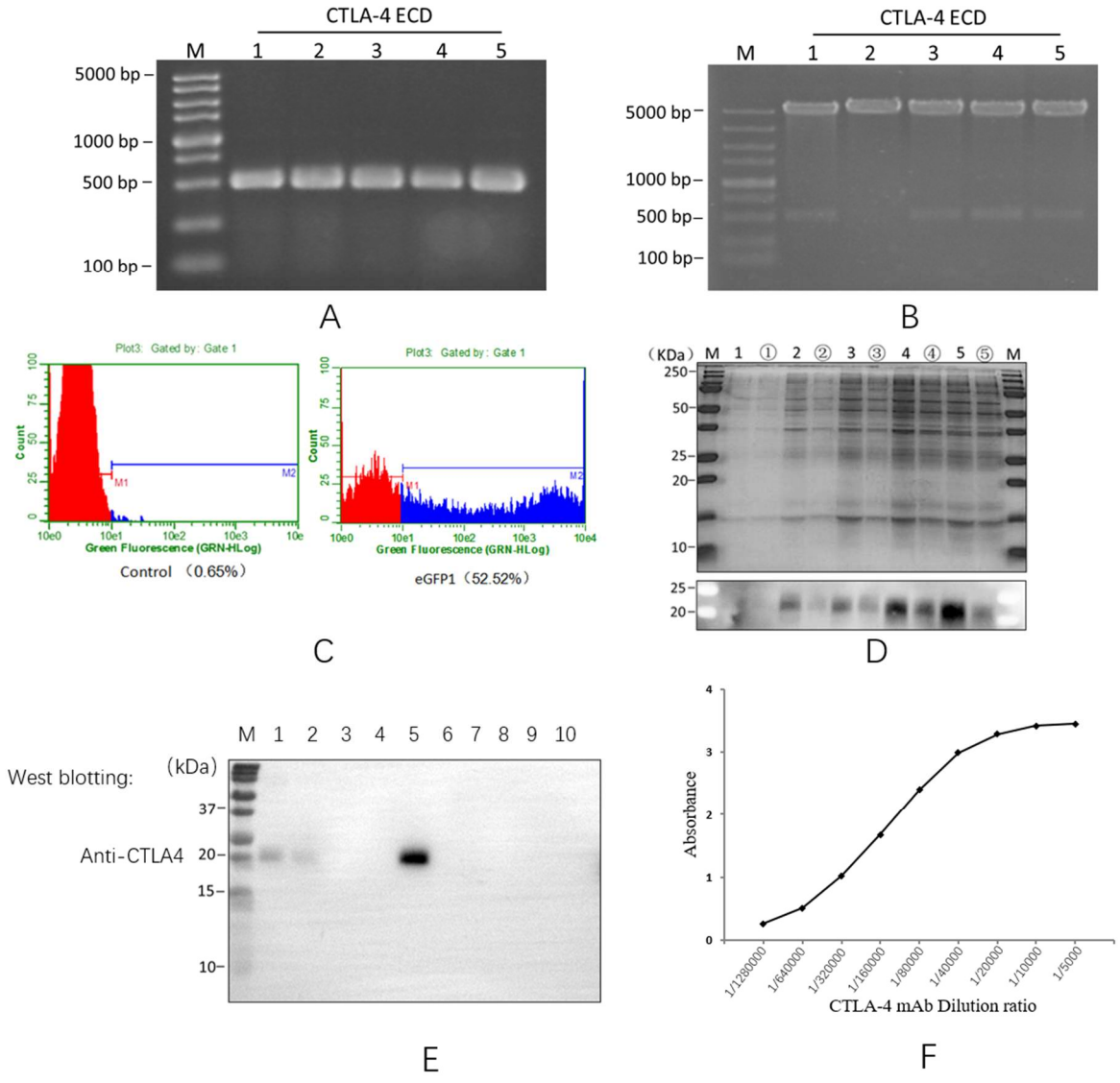

Fig. 1 Expression, purification and identification of extracellular domain of human CTLA-4

From the Guawa flow chart, (fig.1C) the transient efficiency of EGFP was 52.52%, which indicated that the transient condition and process were feasible. The results of WB showed that the expression of pCMV-CTLA-4 ECD in 293F cells was significantly higher than that in pcDNA3.4-CTLA-4 ECD transiently 293F cells, and the expression increased from the first day to the fifth day, and reached the highest level on the fifth day. (fig.1D)

The results showed that the target protein band of CTLA-4 ECD was about 20kD, which indicated that the purification method was feasible and effective. After concentration of 80 mM imidazole elution peak, SDS-PAGE analysis showed that there was a bright band near 20kD. The purity of ImageJ gray analysis was more than 90%, which reached the purity standard of screening nano antibody (fig.1E,F)

According to the fitting curve, the EC50 of the combination of commercial ctla-4mab and CTLA-4 ECD is 140670 times of dilution. By observing the plateau period of "S" curve, it can be concluded that the minimum saturated concentration is 20000 times dilution. These results indicate that the CTLA-4 ECD protein expressed in this project has the biological activity of binding to commercial ctla-4mab, which can be directly used for the elution and screening of nano antibodies. (fig.1G)

3.2 Screening of NbCTLA-4 ECD sequence

The purified CTLA-4 ECD protein was coated overnight on the enzyme plate, and the library capacity was 6.68×1011 CFU/total. After the first round of amplification and three rounds of elution, the library capacity of the phage nanoantibody library was significantly increased. (fig.2A) The enrichment verification results (Fig.2B) showed that the phage content of CTLA-4 ECD was increasing and its specificity was increasing. By ELISA, 18 positive transformants were screened out, and the plasmids were extracted and sent to the company for sequencing. (fig.2C) The molecular weight of the three nanoantibodies were small, and they had the characteristics of small molecular weight of nanoantibodies. The three NbCTLA-4 ECD strains were all hydrophilic soluble proteins with negative gray values. (Table.1)

The purity of NbCTLA-4 ECD was over 93% after concentration, desalination, SDS-PAGE electrophoresis and ImageJ analysis. The antibody production of the three recombinant strains ranged from 4.66 mg/L to 5.84 mg/L, of which G4 was the highest, reaching 5.84 mg/L.(fig.2)

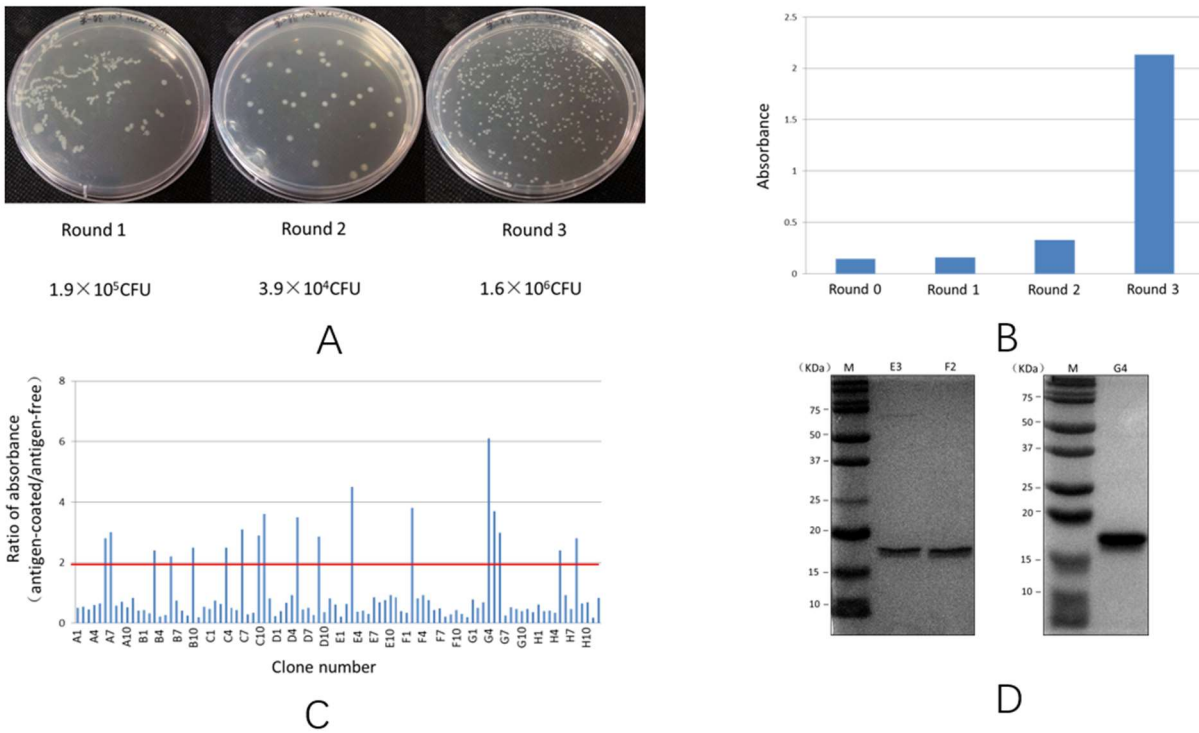

Fig. 2 Screening of NbCTLA-4 ECD sequence

Table 1 Analysis of physical and chemical parameters of NbCTLA-4 ECD

NbCTLA-4 ECD	NW	PI	GRAVY
F2	17382.63	9.3	-0.260
E3	18065.22	8.3	-0.342
G4	17861.79	6.27	-0.359

3.3 Preliminary study on the function of NbCTLA-4 ECD

The three NbCTLA-4 ECD had strong specific binding ability with NbCTLA-4 ECD, and also had certain binding ability with other six control antigens, but the binding ability was relatively weak, among which G4 was the strongest. All the three nano antibodies were determined as anti NbCTLA-4 ECD specific nano antibodies. (fig.3A) Compared with the traditional monoclonal antibodies, NbCTLA-4 ECD has good stability in high temperature treatment, which indicates that they have good thermal stability. In addition, the thermal stability of G4 is higher than that of E3 and F2. (fig.3B) CTLA-4 mAb, F2, E3 and G4 can recognize natural conformation CTLA-4, but none of them has strong recognition ability of CTLA-4. Relatively speaking, G4 is the strongest, E3 is the second, and F2 is the weakest. (fig.3C) However, according to the average fluorescence intensity analysis, compared with the blank control, the three nano antibodies were bound to the cell surface antigen and fluorescence was detected, indicating that the three nano antibodies had certain binding activity. (fig.3D)

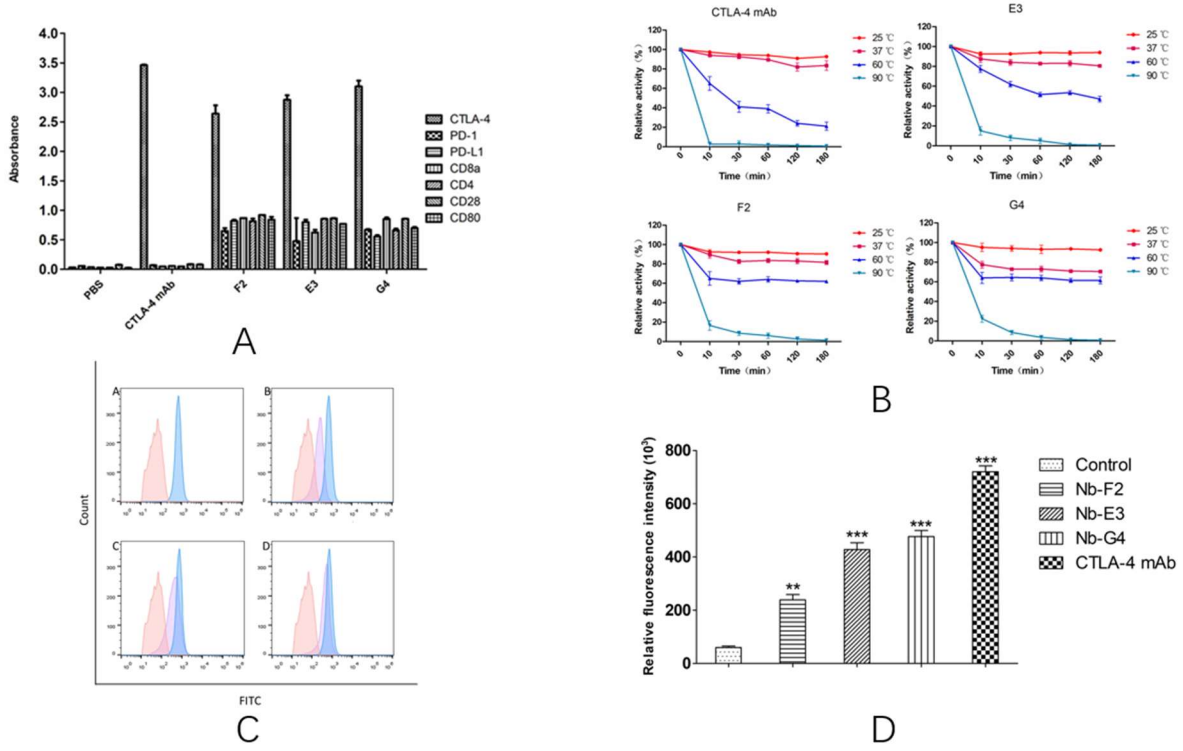

Fig. 3 Preliminary study on the function of NbCTLA-4 ECD

4. Discussion

Recombinant CTLA-4Ig can effectively and specifically inhibit cellular and humoral immune responses in vivo and in vitro, and has significant therapeutic effects on transplant rejection and various autoimmune diseases. It has very low toxicity and side effects, and is considered as a promising immunosuppressive drug. However, there is no CTLA-4 nanoantibody approved for marketing, so the development of CTLA-4 nanoantibody drugs has important scientific significance and application prospects.

The eukaryotic expression vectors pCMV-CTLA-4 ECD and pcDNA3.4-CTLA-4 ECD were constructed by genetic engineering. The pCMV-CTLA-4 ECD expression plasmid was transiently

introduced into HEK293F cells to express CTLA-4 ECD recombinant protein. Finally, the extracellular domain with biological activity and purity more than 90% was obtained. Three strains of NbCTLA-4 ECD binding to the extracellular domain of human CTLA-4 were screened by the first round amplification and multiple rounds of elution using the purified protein as antigen. Although the specificity of the three strains was not as high as that of the positive control, it was mainly bound to CTLA-4 antigen, indicating that the three nanoantibodies still had certain specificity. The results showed that the thermal stability of the McAb was significantly higher than that of the traditional monoclonal antibody at 60 ℃ and 90 ℃. The binding of NbCTLA-4 ECD to CTLA-4 on the cell surface was analyzed. The results showed that the three NbCTLA-4 ECD strains had the ability to recognize CTLA-4 molecules expressed on the cell membrane, but it was weaker than the positive control CTLA-4 mAb, and G4 had the strongest biological binding activity. In conclusion, three strains of NbCTLA-4 ECD (F2, E3, G4) were obtained in this study. Compared with monoclonal antibodies, nanoantibodies are more suitable for use as inhibitors of CTLA-4 because of their unique advantages.

Therefore, this study screened out the CTLA-4 binding nanoantibody through the large-scale phage nanoantibody library, which laid the material foundation for its subsequent application and development.

Acknowledgements

Own founds.

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