

# Production and Evaluation of Specific Single-Chain Antibodies against CTLA-4 for Cancer-Targeted Therapy

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## Abstract

**Background:** Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) molecules are expressed on T-cells and inhibit their function by inhibiting activation of subsequent T-cell molecular pathways. Blocking of CTLA-4 inhibits the growth of malignant tumor cells. Anti-CTLA-4 monoclonal antibodies activate the immune system against cancer. Due to several advantages of single-chain antibodies (scFvs) compared to monoclonal antibodies in cancer immunotherapy, specific anti-CTLA-4 scFvs (single-chain variable fragment) were selected in this study.

**Methods:** A phage antibody display library of scFvs was analyzed and a panning process was performed against an immunodominant epitope of CTLA-4. PCR and DNA fingerprinting were used to differentiate the specific clones. The specificity of the selected clones was investigated by phage ELISA (Enzyme-linked immunosorbent assay).

**Results:** Two specific clones with frequencies of 35 and 20% were identified. The clones reacted with the corresponding epitope on ELISA, while no reactivity was observed with an unrelated peptide, M13KO7 helper phage, unrelated scFvs, or no peptide as negative controls.

**Conclusions:** Targeted therapy against cancer markers is an ideal treatment strategy. Specific human anti-CTLA-4scFvs were selected in this study. These scFvs bound the related epitope. These antibodies have the potential to be used for targeted therapy, where the blocking of CTLA4 receptor is needed. The study suggests further evaluation of the selected scFvs to reveal the effects of the selected antibodies.

**Keywords:** Cancer immunotherapy, CTLA-4, ScFv antibodies

## Introduction

Activation of T-cells for production of immune responses requires two signals from antigen-presenting cells. One signal comes from the major histocompatibility complex (MHC) combined with the antigen, and the other from CD80 (B7.1) or CD86 (B7.2) molecules. T-cell co-stimulation is widely investigated in order to manipulate T-cell reactivity in autoimmune diseases, transplantation, and cancers (1). Activation of T-cells by antigen presenting cells requires co-stimulation between CD28 on the T-cells and B7.1 or B7.2 on the antigen-presenting cells (1). Cytotoxic T lymphocyte-associated antigen 4

(CTLA-4), a second counter receptor for B7.1 and B7.2 on the T-cell surface, binds B7.1 and B7.2 and inhibits T-cell functions by inhibiting activation of subsequent T-cell molecular pathways (2). CTLA-4 has higher affinities for B7.1 and B7.2 than CD28 has, and increases the threshold of signals needed for T-cell activation. T-cell function is decreased by these inhibitory signals from CTLA-4. Blocking CTLA-4 with anti-CTLA-4 antibodies can up-regulate of T-cell function (3). Investigations on CTLA-4-targeted therapy have shown promising strategies for treatment of cancers including melanoma, prostate cancer, renal

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cell carcinoma, non-Hodgkin's lymphoma, colorectal carcinoma, non-small cell lung carcinoma, and breast cancer (4).

Application of monoclonal antibodies against the CTLA-4 antigen is a novel form of cancer immunotherapy (5). Ipilimumab, a human monoclonal antibody against CTLA-4, binds to this marker and blocks the interaction of CTLA-4 with its ligands, B7.1 and B7.2. Blockade of CTLA-4 has been shown to increase T-cell activation and proliferation and has been used to treat late-stage melanoma (6, 7). Abatacept, a fusion protein made from the Fc portion of IgG1 and the extracellular domain of CTLA-4, acts as a selective co-stimulatory modulator and inhibits T-cell activation by binding to B7.1 and B7.2, inhibiting its interaction with CD28 (8). This interaction provides a co-stimulatory signal necessary for T-cell activation (9). Tremelimumab, another humanized monoclonal antibody against CTLA-4, has blocking activity and activates T-cells (10), and is used to treat patients with locally advanced and metastatic melanomas. Treatment with this monoclonal antibody has shown durable objective tumor regression (11).

Despite advantages of monoclonal antibodies, some important problems have been reported; these include high production cost, low tissue penetration, and the human anti-mouse antibody response (HAMA response). Although the synthesized antibodies are humanized, the HAMA response still occurs against the non-human parts of these antibodies (12, 13). Antibody engineering has provided production of small and effective antibodies for cancer immunotherapy. Single-chain antibodies (scFvs), which are composed of variable regions of heavy (VH) and light (VL) chains, are joined by a flexible peptide linker and provide relatively rapid tissue penetration in target tissues when used in high concentrations (14-17). Other benefits of scFvs include high specificities and affinities, low immunogenicities, ease of production, and manipulation possibilities (18-21). Various studies show the effector function of scFvs against the extracellular domain of fibroblast growth factor receptor 3 (FGFR3) (22, 23).

FGFR3 is overexpressed in early stages of bladder cancer and inhibits bladder carcinoma cell line proliferation (23). Inhibition of tumor

angiogenesis by scFvs against vascular endothelial growth factor (anti-VEGF scFvs) has been shown both in vitro and in vivo (24, 25). In this study, specific scFvs against CTLA-4 were selected using a phage display technique, and the specificities of the selected scFvs were evaluated by ELISA.

## Materials and Methods

### *Phage Rescue*

An scFv phage antibody library was produced, as described previously (26, 27). The *Escherichia coli* (*E. coli*) -containing phagemid was cultured on 2TYG agar/ampicillin (tryptone, yeast extract, glucose, agar, and ampicillin) (Merck, Germany) plates overnight at 30 °C. The cells were scraped and incubated in 2TYG broth at 37 °C for 1 hr. M13KO7 (kanamycin resistant helper phage) was added and the cells were incubated with shaking at 37 °C for 30 minutes. The cells were centrifuged at 3500 rpm for 20 min. The bacterial pellet was transferred to 2TY broth containing ampicillin and kanamycin and incubated with shaking at 30 °C overnight. The supernatant of the centrifuged culture was passed through 0.2 µm filters and stored at 4 °C.

### *Panning process*

Polystyrene immunotubes were coated with the peptide MHVAQPAVVLA (28) (Nunc, Denmark) and incubated at 4 °C overnight. The tubes were blocked with 2% skimmed milk and incubated at 37 °C for 2 hrs. The tubes were washed four times with phosphate-buffered saline (PBS)/Tween20 and four times with PBS. The phage supernatant was added to the tubes and incubated at room temperature for 1 hr. Logarithmic phase *E. coli* cells were added, incubated at 37 °C for 1 hr, and then centrifuged at 3500 rpm. The bacterial pellet was plated on 2TY agarose/ampicillin plates and incubated at 30 °C overnight. Four rounds of panning were performed to select epitope-specific scFv antibodies.

### *DNA Fingerprinting of the Selected Clones*

The inserts of the selected clones were amplified by PCR. To show the fingerprinting patterns, 17 µl of the PCR products were digested with Mva-I (Roche Applied Science, Germany) and electrophoresed in a 2% agarose gel.

**Phage ELISA**

Wells of a polystyrene plate were coated with the peptide and incubated overnight at 4 °C. After washing with PBS, wells were blocked with 2% skimmed milk and incubated at 37 °C for 2 hrs. Phage rescue supernatant containing the appropriate scFv, diluted 1:1 with blocking solution, was added to each well and incubated at room temperature for 2 hrs. To remove unbound phage, the wells were washed three times with PBS/Tween20, and three times with PBS. Anti-Fd bacteriophage (Sigma, Germany), an antibody produced in rabbit which is used in ELISA for antiphage immunohistochemistry, was added to each well and incubated for 1 hr. After washing, horseradish peroxidase (HRP) -conjugated anti-rabbit antibody (Sigma, Germany) was added and incubated at room temperature for 1.5 hrs. The wells were washed and citrate buffer containing azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) (Sigma-Aldrich, Germany) and H<sub>2</sub>O<sub>2</sub> were added. Phage ELISA was done for three times and the absorbance was read at 405 nm on an ELISA reader. The wells without peptide, with unrelated

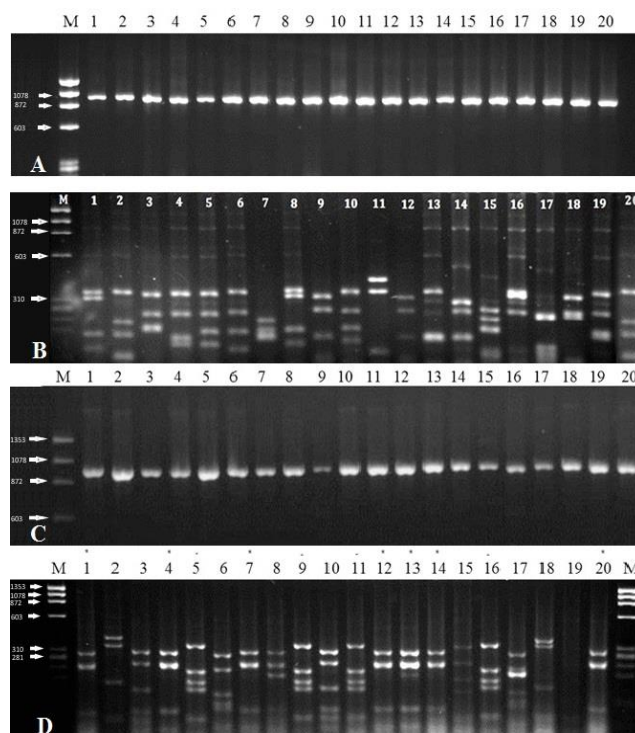
peptide (prostate stem cell antigen peptide), unrelated scFv (scFv to HER2), and M13KO7 helper phage, were included as negative controls.

**Results**

**PCR and DNA Fingerprinting**

Figures A and B show PCR and DNA fingerprinting of 20 clones before panning process, respectively. PCR products of 950 bps (VH-linker-VL) were amplified from all the isolated clones before panning (Fig. A). Products of the corresponding Mva-I digests are shown in Figure B.

PCR products of 950 bps were also amplified from all the isolated clones after panning (Fig. C). The corresponding Mva-I digests of those PCR products are shown in Figure D. Two dominant fingerprint patterns (specific repeated designs) were obtained after panning. Clones with pattern 1, scFv1 (Lanes 1, 4, 7, 12, 13, 14, and 20), with a frequency of 35%, and pattern 2, scFv2 (Lanes 5, 9, 11, and 16), with a frequency of 20%, were selected.



**Fig. 1.** (A). Agarose gel electrophoresis of the PCR products of the selected clones before panning. A 950 bp band was amplified from each clone. M:  $\Phi$ X174DNA marker. (B). Agarose gel electrophoresis of Mva-I digests of the PCR products before panning. (C) Agarose gel electrophoresis of the PCR products of the selected clones after panning. A 950 bp band was amplified from each clone. M:  $\Phi$ X174DNA marker. (D) Agarose gel electrophoresis of Mva-I digests of the PCR products after panning. Pattern 1, scFv1, is marked with a star (Lanes 1, 4, 7, 12, 13, 14, and 20), and pattern 2, scFv2, is marked with negative sign (Lanes 5, 9, 11, and 16).

**Phage Enzyme Linked Immunosorbent Assay (Phage ELISA)**

Phage ELISA was used to demonstrate the specific binding of the selected scFv antibodies to the corresponding peptide. The absorbances of the wells coated with the corresponding peptide for the two

selected scFvs were greater than those of the wells containing no peptide. Optical densities (ODs) of 0.66 and 0.59 were obtained for the reactions of scFv1 and scFv2 with their corresponding epitopes, while ODs less than 0.2 were obtained with all the negative controls. (Fig. 2).

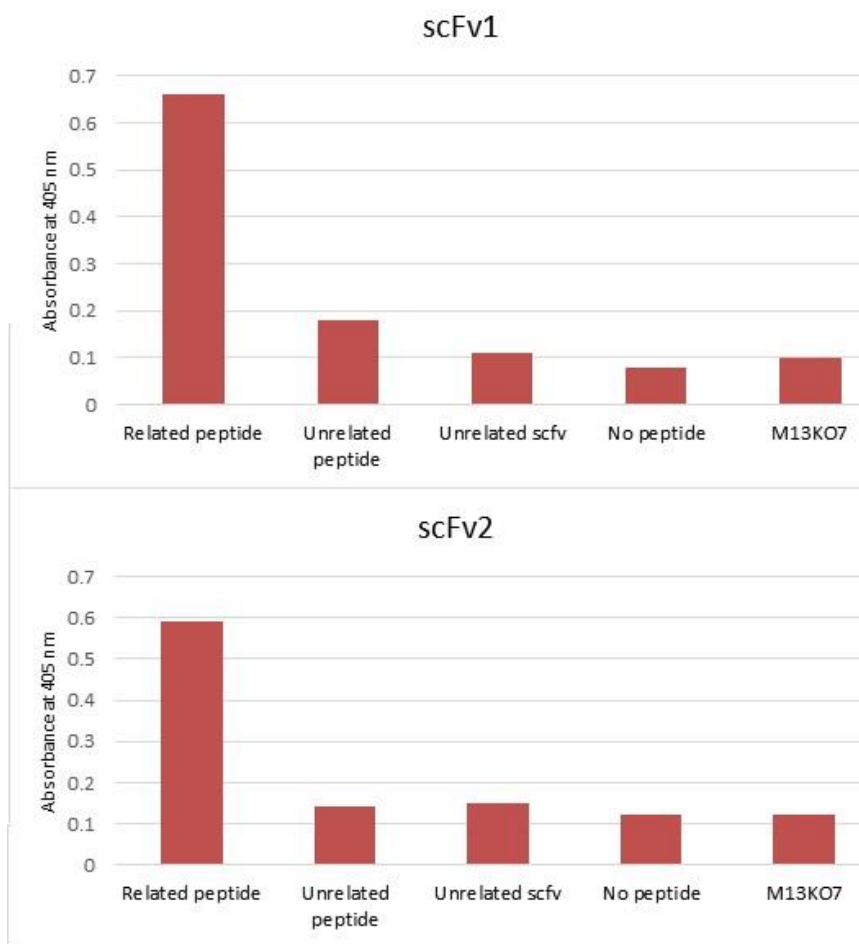


Fig. 2. ELISA results of scFv1 and scFv2.

As Thathaisong showed (29), if the absorbance of selected scFv is more than 2 times greater than the absorbance of the negative controls there is significant difference between them, so there is no need for further statistical analysis. In this study the absorbance of the wells coated with mentioned peptide for the selected scFv1 and scFv2 was 8.25 and 4.9 folds higher than that of the negative control wells, respectively.

**Discussion**

Although cancer treatment has progressed greatly in recent decades, most treatments have disadvantages. Radiotherapy and chemotherapy

are not targeted and affect both normal and malignant cells. These methods also have numerous side effects including sore skin, nausea, loss of appetite, diarrhea, infertility, hair loss, lymphedema, and blood and nervous system disorders, many of which are irreversible. In addition, chemotherapy drugs have limited efficacy (30). Monoclonal antibody treatment offers improved results compared to previous methods, but some disadvantages and rejection in humans has limited their clinical use (31, 32). ScFv treatment has been introduced as one of the best cancer immunotherapies due to the human origin

of the antibodies and their small sizes and tissue penetration abilities (32).

Because inhibition of CTLA-4 to B7 binding is critical in cancer treatment, blocking CTLA-4 may be an effective immunotherapy (33). In this study, specific scFvs were selected against CTLA-4. The amino acid sequence MHVAQPAVVLA, located in the N-terminus of CTLA-4, was used as the epitope. This epitope has been reported as an immunodominant CTLA-4 epitope, and a human monoclonal antibody produced against it inhibited tumor growth in a murine fibrosarcoma *in vivo* (28, 34).

A panning process was applied to select specific scFvs against CTLA-4. DNA fingerprinting of the library identified two specific scFvs with frequencies of 35% and 20%. The panning process has been used to select specific scFvs against different targets. To select specific scFvs against the extracellular domain of FGF3, which has a significant role in bladder carcinoma cell line proliferation (22), a library was panned against the receptor and the antibodies were selected (23).

Five specific antibodies against MUC1, an antigen overexpressed in ovary adenocarcinoma, were selected, and their relative reactivities analyzed by phage ELISA (35). ScFvs against a specific epitope of P185, a phosphoglycoprotein that is overexpressed in most cancers of epithelial origin and increases tumor aggressiveness, were isolated and their specificities evaluated by phage ELISA (27).

In the current study, the panning results were confirmed by ELISA. The ELISA results showed that the two selected scFvs bound to the corresponding peptide with greater affinities than the negative controls. Moreover, the epitope was not detected by the antibody controls, unrelated scFv, or M13KO7. The unrelated scFv to HER2 did not react with the CTLA-4 peptide. The absorbances of the wells coated with the CTLA-4 peptide for scFv1 and scFv2 were 8.25 and 4.9 - fold higher than that of the wells containing no peptide, respectively. The specificity of the selected scFvs to the corresponding peptide has been shown in various phage ELISA assessments. The optical density (OD) of specific scFvs against influenza-A virus H5N1 subtype at 405 nm were shown by Thathaisong et al. to be two-fold greater

than the negative controls in a positive phage ELISA (29). There was no reaction with an unrelated peptide and the result represented the specific reaction of the selected scFvs with the corresponding peptide.

CTLA-4 is expressed on many cancerous cells and is introduced as a potential marker for immunotherapy (33, 36). Several antibodies against CTLA-4 are under study in clinical trials. Tremelimumab is currently in phase II of a clinical trial and being investigated for melanoma and malignant mesothelioma treatments (37). Ipilimumab is in phase III of a clinical trial and has shown promise against melanoma and prostate and lung cancers (37, 38). Due to several advantages of scFvs over full-length antibodies, these small and high-affinity human antibodies have been applied for cancer-targeted therapy. scFvs against EpCAM, HER2, IL-25, and CEA have been reported as effective against cancer cells (39-41). Also, immune-conjugated forms of specific scFvs, including immunocytokines against ganglioside (GD2) (42), anti-tumor associated antigen (TAA) photodynamic or sonodynamic (PS) -conjugated antibody (43), and anti-VEGFR-2scFv conjugated with As2O3-stealth nanoparticles (44), have shown promise against tumor growth and angiogenesis. The specific anti-CTLA-4 scFvs selected in this study are new agents with promise for targeted therapy of cancers expressing CTLA-4. The antibodies could interfere with cellular process by binding to the extracellular portion of CTLA-4. These antibodies originate from human immunoglobulin and do not induce a HAMA response. In addition, the small sizes and high affinities of the selected scFvs contribute to efficient penetration and effective functions than routine monoclonal antibodies. Also, conjugated antibodies can be produced to exert additional anti-cancer effects. Further investigations are needed to evaluate the anti-tumor effects of the selected antibodies *in vitro* and *in vivo*.

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