Anti-CTLA4 scFv, a Single—Chain Antibody, Can Prolong Expression of *Escherichia. coli.* beta-Galactosidase in Immune—Competent Mice

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ABSTRACT

Objectives: To induce immune tolerance in immune-competent animals with a plasmid (p4F10-eB7) expressing a membrane-bound single-chain antibody against CTLA4. **Materials and Methods:** Twenty 9-week old immune-competent C57B/L6 mice were divided into 2 groups. Ten mice received an intramuscular injection of 50 μL of endotoxin-free pCBLacZ plasmid at a concentration of 1 mg/mL in phosphate buffer in the left anterior tibialis. The plasmid codes for the potent immunogen, *Escherichia coli* beta-galactosidase in transduced muscle fibers. Ten mice received a co-injection of p4F10-eB7 with pCBLacZ. **Results:** We examined the expression of beta-galactosidase in muscle fibers on the 6th and 12th days after the intramuscular injection. The average number of fibers expressing beta-galactosidase was 43.6 in the mice receiving 2 plasmids as compared with 2.2 in the mice receiving only pCBLacZ (p= 0.015, by the Mann-Whitney test). There was no significant difference in the expression of beta-galactosidase between these 2 groups on day 12, at 0.4 Vs 1.4 fibers (p=0.419). **Conclusions:** Expression of anti-CTLA4 antibody in muscle cells prolonged the expression of a strong immunogen, *E. coli* beta-galactosidase in transduced muscle fibers of immune-competent mice. The probable mechanism could be anergy of activated cytotoxic T cells induced by activation of CD152 after interaction with the single-chain antibody. (***Tzu Chi Med J*** 2004; **16**:25-31)

Key words: immunomodulation, transgene, T cell receptor, single-chain antibody

INTRODUCTION

Gene therapy is a potential treatment for many acquired and hereditary diseases. Besides transduction efficacy, tissue-specific targeting, and stable expression of the transgene, the host immune responses play a crucial role in success of gene therapy [1]. Host immune responses can be induced not only by vector gene products, but also by transgenic proteins [2-4]. Immunosuppressive agents such as steroids and FK506 have been used to attenuate host immunity and prolong the expression of transgenes [5-7]. Systemic side effects, including increased probability of opportunistic infection and reactivation of chronic infection, are drawbacks of these methods.

Tolerance induction by targeting recipient T cells can prevent the host from rejecting donor grafts [8]. Activation of T cells is a 2-step process. The first step is antigen specific and involves interaction between T cell receptors and antigenic peptides, in association with MHC on the surface of antigen-presenting cells (APCs). This interaction is specific, but not sufficient for full T cell activation. The second step is antigen independent,

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but requires B7-1 and B7-2 membrane proteins on the professional APCs. This interaction between CD28 on the surface of T cells and B7-1 or B7-2 on APCs is a potent second signal for T cell activation [9-11]. These costimulatory signals provoke release of cytokines and proliferation of activated cells. Immunomodulational approaches that target the ligand-ligand interaction have produced successes in some preclinical gene therapy trials [12]. For example, blockade of the interaction between B7s and CD28 by a soluble CTLA4 fusion protein, CTLA4-Ig, inhibits T cell activation and leads to anergy and induction of tolerance *in vitro* and *in vivo* [13-16]. Another inducible T cell receptor, CD152 (CTLA4, cytotoxic T cell-associated antigen 4), plays a role in modulating T cell activation. CD152 on activated T cells binds to B7-1 or B7-2 on APCs to induce apoptosis of these cells and decrease the release of cytokines. Therefore, activation of CD152 with a membrane-immobilized single-chain anti-CTLA4 scFv has been applied in some preclinical trials [17-19].

Surface expression of proteins can decrease systemic side effects and efficiently elicit biological activities of enzymes, single-chain antibodies, and cytokines. Immunoglobulins consist of 4 polypeptide chains, 2 heavy and 2 light chains held together by disulphide bounds. Artificial single-chain antibodies (scFv) composed of the variable regions of 1 heavy and 1 light chain possess similar antigen specificity and potency as compared with natural immunoglobulins. We recently constructed 2 plasmids encoding anti-CD3 and anti-CTLA4 scFv for immunomodulation [20]. These plasmids can express immobilized proteins on the membrane of mammalian cells [21]. To test the efficacy of the immune tolerance induced by anti-CTLA4 scFv *in vivo*, we used *E. coli* beta-galactosidases as an immunogen [22,23]. Two plasmids, 1 encoding surface anti-CTLA4 single chain antibody and the other expressing beta-galactosidase, were co-injected in the left anterior tibialis of adult immune-competent mice. Tolerance induction was monitored by expression of this foreign protein in muscle fibers.

MATERIALS AND METHODS

Animals

We performed all animal care and experiments according to the guidelines of animal care of the Animal Center of Tzu Chi University. Adult C57BL/6 mice were purchased from the National Laboratory Animal Breeding and Research Center, Taipei, Taiwan and bred in a specific-pathogen-free environment. Twenty 9-week old adult mice were divided into 2 groups. The first group received an intramuscular injection of 50 μL of pCBLacZ at a concentration of 1 mg/mL in phosphate buffer in the left anterior tibialis. The other group received co-administration of the plasmid, p4F10-eB7, in the same volume and same concentration in the left anterior tibialis. Half of the mice in each group were sacrificed by an intraperitoneal injection of urethane (0.002 mL/g body weight, Sigma, St. Louis, MO) after 6 days, and the other half were sacrificed after 12 days.

Construction of plasmids

The plasmid, pCBLacZ, was constructed by excising the *E. coli* beta-galactosidase gene from the plasmid, pCMV-beta (BD Bioscience Clontech, Palo Alto, CA), with *Not I*, blunting with Klenow polymerase and then adding *Xho* I linkers at both ends. The modified DNA fragment was cloned into the *Xho I* site of pCAGGS (a kind gift from Prof. Miyazsaki, Osaka University, Osaka, Japan). This plasmid, composed of a potent hybrid promoter containing a chicken beta-actin promoter and CMV early enhancer, has been used for a long time in gene expression studies.

To construct p4F10-eB7, the IgG-like type C domain, the transmembrane domain and cytoplasmic tail of murine CD80 was RT-PCR-amplified from pCD80/zeo (kindly provided by Dr. Gordon Freeman, Dana-Farber Center Institute, Harvard Medical School, Boston, MA). The PCR product was cut with *Sal I* and *Xho I* and inserted into p2C11-B7 [20] in place of the B7 transmembrane domain and cytoplasmic tail to obtain p2C11-eB7. The anti-CTLA-4 single-chain antibody was cloned from total RNA isolated from UC10-4F10-11 [24] hybridoma cells (American Type Culture Collection, Manassas, VA) following the method published by Gilliland and colleagues [25]. Leucine residues at positions 43 and 89 of the 4F10 variable light chain sequence were mutated to methionine and glutamine, respectively, to increase scFv surface expression [26]. This membrane-immobilized scFv contains a chimeric receptor composed of a murine immunoglobulin k chain signal peptide, an influenza HA epitope, anti-CTLA-4 scFv, followed by the Ig-like C2-type and Ig-hinge-like domains, transmembrane and cytosolic tail of murine B7-1.

Preparation of endotoxin-free plasmids for intramuscular administration

Plasmid DNA was purified with the QIAGEN ENdoFree Plasmid Maxi Kit (QIAGEN, Valencia, CA) and dissolved in endotoxin-free distilled water. The plasmid was diluted to 1 mg/mL in phosphate buffer before

injection [27].

In vivo expression of the recombinant, scFv anti-CTLA4 scFv

The anti-CTLA4 scFv contains an HA epitope near its N-terminus. *In vivo* expression of anti-CTLA4 scFv was therefore evaluated by immunochemical staining of transduced cells with anti-HA. Muscle cryosections were rinsed with phosphate-buffered saline (PBS) at room temperature for 15-30 min, and the endogenous peroxidase was blocked with peroxidase blocking reagent (DAKO, Carounteria, CA) for 5 min at room temperature. The slides were rinsed in PBS, blocked with 10% goat serum in PBS for 1 hour, and then incubated with rat anti-HA antibody (Roche Applied Science, Indianapolis, IN) overnight at room temperature. Sections were rinsed with 10% goat serum in PBS and then incubated with biotinylated goat anti-rat secondary antibody for hour hour. Sections were next rinsed with 10% goat serum in PBS as before, and incubated with avidin DH and biotinylated horseradish peroxidase H reagents (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA) for 30 min. After that, the slides were rinsed with PBS, and peroxidase activity was localized with diaminobenzidine tetrahydrochloride (DAB substrate kit for peroxidase, Vector Laboratories).

In vivo expression of *E.coli* beta-galactosidase in muscles

The anterior tibialis muscle was divided into 4 consecutive regions. Each region was cryosectioned at 10-mm thickness. Five slices were taken from each region for serial histochemical and immunochemical studies. For checking expression of *E. coli* beta-galactosidase, slices were rinsed with PBS two times at room temperature for 15-30 min and then stained with 0.1% X-Gal, 5-bromo-4-chloro-3-indolyl beta-D-galactoside (Sigma, St. Louis, MO) in 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 2 mM $MgCl_2$ at 37˚C overnight. After checking the expression of beta-galactosidase, muscle slices were stained with hematoxyllin and eosin to determine the total number of muscle fibers in each cross-section. Expression of beta-galactosidase was quantified as the total number of blue-stained fibers in a series of consecutive muscle slices of the sampled muscle.

Detection of $CD4^+$ T cells in muscle cryosections

Procedures for the immunochemical staining of $CD4^+$ cells were similar to those for detection of the influenza HA epitope as described above. The primary antibody we used to detect CD4 was rat anti-mouse CD4 (PharMingen, clone No: RM4-5).

Statistical analysis

Data were analyzed using nonparametric analysis, with the Mann-Whitney test. Statistical significance was set at $p < 0.05$.

RESULTS

Anti-CTLA4 scFv was detected 6 days after the intramuscular injection of p4F10-eB7 (Fig. 1), but could not be detected on the 12th day. Fewer blue-stained muscle fibers (2.2 Vs 43.6) in the control group as compared with mice the received co-injection of anti-CTLA4-scFv (Table 1A, Fig. 2) (p=0.015, by the Mann-Whitney test). There was no significant difference between these 2 groups 12 days after intramuscular injection (Table 1B). To evaluate cellular immunity, we examined infiltration of $CD4^+$ T cells in muscles 6 days after the intramuscular injection (Fig. 3). There was no obvious infiltration of $CD4^+$ cells in mice receiving pCBLacZ (panels 1.1~1.5). Four slices showed prominent T cell infiltration in mice that received the co-injection of anti-CTLA4 scFv plasmid (panels 2.1, 2.3, 2.4 and 2.5). We could find no obvious T cell infiltration in either group 12 days after the intramuscular injection. Although the mice receiving anti-CTLA4 scFv had more-prominent infiltration of $CD4^+$ cells, hematoxylline and eosin staining revealed more-severe muscle fiber destruction and inflammatory cell infiltration in mice that received only beta-galactosidase (Fig. 2, panels B and D).

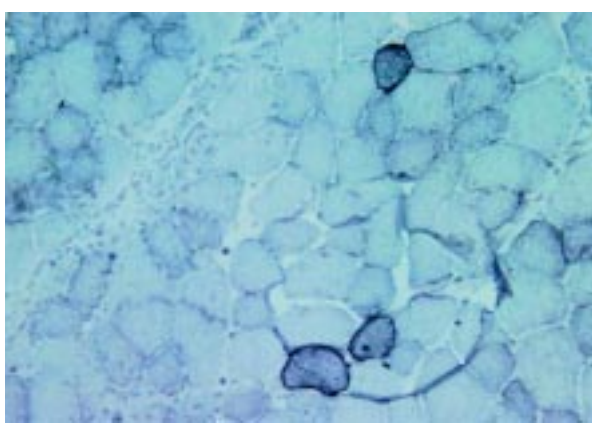

Fig. 1. *In vivo* expression of anti-CTLA4 scFv demonstrated by immunochemical staining for influenza hemagglutinin on the muscle membrane 6 days after intramuscular injection.

Table 1. Muscle fibers expressing *E. coli* beta-galactosidase after intramuscular injection of the plasmids

A. Sixth Day after Injection

Mouse	1	2	3	4	5	Mean	p*
pCBLacZ	1	0	1	8	1	2.2	0.015
pCBLacZ +anti-CTLA4-scFv	14	7	15	84	98	43.6	

B. Twelfth Day after Injection

Mouse	1	2	3	4	5	Mean	p*
pCBLacZ	5	1	0	1	0	1.4	0.419
pCBLacZ + anti-CTLA4-scFv	1	0	1	0	0	0.4	

Number of blue fibers after being incubated with 5-bromo-4-chloro-3-indolyl-b-D-galactoside;
The results are presented as average number of blue fibers in cryosections from 5 consecutive regions;
*: By Mann-Whitney Test

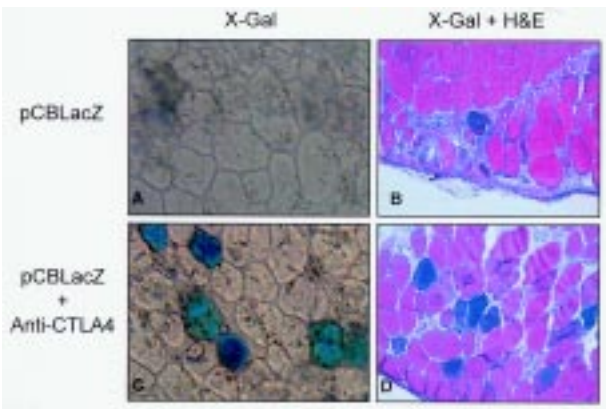

Fig. 2. *In vivo* expression of *E. coli* beta-galactosidase 6 days after intramuscular injection. Panel B shows marked destructio and inflammatory cell infiltraion around the transduced muscle fibers in the animal receiving pCBLacZ injection only.

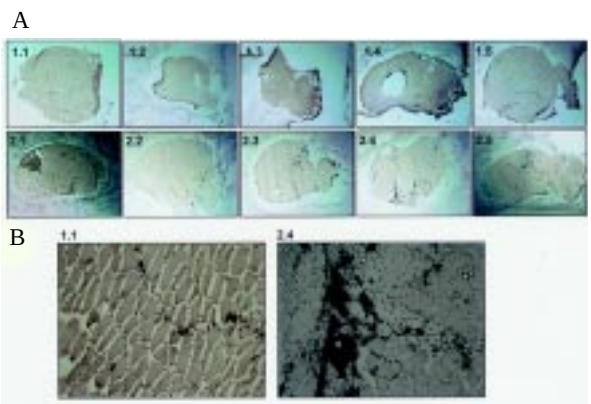

Fig. 3. $CD4^+$ T cells infiltration shown by immunochemical staining 6 days after injection of plasmid DNA. No obvious infiltration was found in the mice receiving beta-galactosidase, panel 1.1~1.5. The animals received anti-CTLA4 scFv and betagalactosidase plasmids had prominent $CD4^+$ T cell infiltration at the injection sites, except number 2, panel 2.1~2.5. Panel B shows close-up views of the muscle fibers.

DISCUSSION

The expression of beta-galactosidase in muscle fibers was higher in animals that received a coinjection of anti-CTLA4 scFv as compared with their littermates on the 6th day after the intramuscular injection of beta-galactosidase. Only 1 mouse in the control group displayed 2 blue fibers 6 days after injection, whereas all mice that received anti-CTLA4 scFv with beta-galactosidase plasmid DNA displayed multiple blue fibers (Table 1). Two mice had more than 80 blue fibers in the study group. One of the problems of intramuscular gene delivery is the heterogeneity of gene expression. Leakage of phosphate buffer from the injection site was noted during this study. In addition, destruction of DNA in the interstitial space by DNase is another probable cause of heterogeneous expression. Because of the heterogeneous expression of beta-galactosidase, we used non-parametric analysis for our results. The Mann-Whitney test showed a significant difference between these 2 groups, at p=0.015, on the 6th day after the injection.

Muscle fibers expressing beta-galactosidase could not be detected in either group 12 days after injection of DNA. This could have been due to a shutting down of the promoter, or to anti-CTLA4 scFv being able to protect the immunogen but not being strong enough for a long-term study. This is because these 2 genes, beta-galactosidase and anti-CTLA4-mAb, are regulated by 2 different promoters, a potent hybrid promoter, the CB promoter, and a human CMV promoter, respectively.

It is believed that CTLA4 plays a major role in the induction and maintenance of peripheral T cells tolerance [28]. Fig. 3 shows that infiltration of $CD4^+$ T cells was more prominent in the study group as compared with the control group. This result differs from a recent report in which a soluble recombinant protein, CTLA4Ig, was employed to block interaction between B7 and CD28 for the induction of immune tolerance [14]. Infiltration of $CD4^+$ cells was less prominent in mice receiving this immunomodulator in their study. Because the expression of CD4 is also expressed by regulatory T cells, T_{Reg}, that co-express CD25, it has been shown that activation of CTLA4 induced proliferation of T_{Reg} cells and peripheral immune tolerance [28-31]. We propose that membrane-bound anti-CTLA4 scFv and soluble anti-CTLA4-Ig may mediate different pathways to induce immune tolerance.

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單鏈抗體anti-CTLA4-scFv能延長大腸桿菌半乳糖酶在免疫力正常的小黑鼠體內表達

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摘要

目的：本研究之目的是要探討以基因轉植的方法在肌肉細胞表達毒殺 T 細胞相關第四抗原單鏈抗體－ anti-CTLA4 scFv以達到誘發免疫耐受性之可能。**材料與方法**：我們將九週大且具正常免疫力的小黑鼠，C57B/L6分成兩組每組十隻，對照組在其左前脛骨肌注射以溶於磷酸鹽緩衝溶液不含細菌內毒素的質體－pCBLacZ，DNA 的濃度為 1 mg/mL ，注射劑量為 50 微升(μL)，這個質體將表達大腸桿菌半乳糖酶以誘發局部的免疫反應。實驗組則除了注射pCBLacZ外還注射相同濃度相同劑量的另一質體－p4F10-eB7，這個質體可以在肌細胞膜表達anti-CTLA4 scFv。**結果**：我們在注射後第六和第十二天檢視小鼠的肌肉切片，發現呈現半乳糖酶活性的肌細胞數在第六天時，對照組平均為 2.2 ，以無母數分析 Mann-Whitney test 得 p 值為 0.015；實驗組為 43.6；在第十二天時兩組則沒有顯著的差異，分別為1.4 與0.4， p值為0.419。**結論**：單鏈抗體－anti-CTLA4 scFv，可以延長外來蛋白－大腸桿菌半乳糖酶在正常免疫力小黑鼠的肌肉中表達，其作用機轉可能是作用在已活化的 T 細胞表面上的受器－ CD152 ，藉活化 CD152 使得 T 細胞凋亡，進入 anergy ，達到降低宿主免疫反應的目的。(**慈濟醫學** 2004; **16**:25-31)

關鍵語：免疫調控、轉植基因、T 細胞受器、單鏈抗體

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