Anti-CTLA4 scFv, a Single-Chain Antibody, Protects the Expression of *E. coli.* beta-Galactosidase during Repeated Intramuscular Injections in Immune-Competent Mice

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ABSTRACT

Objectives: To evaluate whether anti-CTLA4 scFv, a membrane-bound CD152 activator, could protect the expression of a strong immunogen, *E. coli* beta-galactosidase, during repeated intramuscular injections in immune-competent animals. **Materials and Methods:** Ten six-to-nine-week old immune competent C57BL/6 mice were divided into two groups. Mice in the first group received four consecutive intramuscular injections of 50 μL endotoxin-free pCBLacZ plasmid in the left anterior tibialis at intervals of one week.The plasmid coded a potent immunogen, *E. coli.* beta-galactosidase. The other five mice received co-injections of p4F10-eB7, which expressed anti-CTLA4 scFv, and pCBLacZ. All the mice were sacrificed 6 days after the last intramuscular injection. **Results:** We examined the expression of beta-galactosidase in muscle fibers. The average number of fibers expressing beta-galactosidase was 12.6 in the mice receiving two plasmids compared with 1.4 in their littermates. There were obvious endomyosial and perimyosial infiltrations of inflammatory cells in both groups. **Conclusions:** Repeated intramuscular injections of beta-galactosidase can induce strong immune responses in immune-competent animals and cause eradication of transduced muscle fibers by inflammatory cells. Co-administration of anti-CTLA4 scFv can protect the transduced cells. This single chain antibody may be used to prolong the expression of transgene during repeated intramuscular gene delivery. (***Tzu Chi Med J*** 2006; **18**:259-265)

Key words: transgene, T-cell receptor, single chain antibody, immune responses, CTLA4

INTRODUCTION

Muscle targeting gene therapy is a potential treatment not only for hereditary muscle diseases, such as Duchenne muscular dystrophy, but also for many other acquired and hereditary diseases, such as hemophilia, Pome's disease and hematopoietin deficiency in uremic patients. Besides transduction efficacy and stable expression of the transgene, the host immune responses play a crucial role in the success of gene therapy [1]. Host immune responses are induced not only by vector gene products and CpG motifs but also by transgenic proteins [2-5]. It has been shown that the mutant hosts develop immune responses to the transgenic products that are non-immunogenic in normal animals [6].

Repeated intramuscular injections of genetic vectors are necessary to maintain a long-term expression and meet the requisite efficacy in clinical application. However, repeated intramuscular gene delivery may induce a stronger host immune response than a single application dose. Immunosuppressive agents such as

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steroids, cyclosporine, and FK506 have been used to attenuate host immunity and prolong transgene expression [2,7-9]. Systemic side effects, including increased probability of opportunistic infections, development of cancer which escapes host immunity and reactivation of chronic infection, reduce the benefits of these methods.

Tolerance induced by targeting recipient T cells can prevent donor graft rejection by the host [10]. Several methods including drugs, cytokines, and recombinant proteins have been used to induce peripheral immune tolerance [11]. The lack of antigen-specificity is one of the drawbacks of these strategies. We have demonstrated that co-administration of a plasmid encoding a membrane bound single chain antibody, anti-CTLA4 scFv, can protect the expression of a strong immunogen, *E. coli* beta-galactosidase, in muscle cells in immune competent animals [12]. Herein, we would like to show that this single chain antibody can also protect transduced muscle fibers during repeated intramuscular injections.

MATERIALS AND METHODS

Animals

We performed all animal care and experiments according to the guidelines on animal care of the Animal Center at Tzu Chi General Hospital. Adult C57BL/6 female mice aged from 6 to 9 weeks were purchased from the National Laboratory Animal Breeding and Research Center, Taipei, Taiwan. Animals were bred in a specific pathogen-free facility. Ten adult female C57BL/6 mice were divided into 2 groups. All the mice received 4 consecutive intramuscular injections of endotoxin-free plasmid dissolved in 50 μL phosphate buffer in the left anterior tibialis at intervals of one week. Animals in the study group received 50 μg pCBLacZ and the same amount of p4F10-eB7. Their five littermates received 50 μg pCBLacZ alone. Six days after the last intramuscular injection, the animals were humanely sacrificed using an intraperitoneal injection of urethane (0.002 mL/g body weight, Sigma, St. Louis, MO). The left anterior tibialis were isolated and frozen in pre-chilled isopentane, and then kept in a -80°C freezer. The sera were collected by cardiac puncture, isolated by centrifugation and then kept in a -20°C freezer prior to analysis.

Construction and endotoxin free preparation of the plasmids

We used two plasmids, pCBLacZ and p4F10-eB7 in this study. The former is an expression cassette of *E. coli* beta-galactosidase driven by a strong hybrid promoter composed of chicken beta-actin promoter and human CMV early enhancer. The latter encodes a recombinant membrane bound single chain antibody, anti-CTLA4 scFv, which can activate CD152 on activated lymphocytes. Details of the construction of the plasmids and procedures for endotoxin-free preparation were published in our previous paper [12].

In vivo expression of beta-galactosidase

The anterior tibialis muscle was divided into 4 equal sections, by length. Each region was cryosectioned into 8 mm thick slices. Five slices were taken from each region for serial histochemical and immunochemical studies. To check the expression of *E. coli* beta-galactosidase, slices were rinsed with phosphate-buffered saline (PBS) three times at room temperature for 30 minutes and then stained with 2% 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 for two hours. After checking the expression of beta-galactosidase, muscle slices were stained with hematoxyllin and eosin to determine the total number of muscle fibers and the pathological changes in these fibers. Expression of beta-galactosidase was quantified as the maximal number of blue-stained fibers in the four sampled regions of the muscle.

In vivo expression of recombinant anti-CTLA4 scFv

The anti-CTLA4 scFv contains a hemagglutinin epitope near its N-terminus. *In vivo* expression of anti-CTLA4 scFv was therefore evaluated by immunochemical staining of transduced cells with anti-HA monoclonal antibody. Muscle cryosections were rinsed with PBS at room temperature for 15-30 min, and the endogenous peroxidase was blocked with peroxidase blocking reagent (DAKO, Carpinteria, 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, USA) overnight at room temperature. Sections were rinsed with 10% goat serum in PBS and then incubated with biotinylated goat anti-rat secondary antibody for one hour. Sections were next rinsed with 10% goat serum in PBS, and incubated with avidin DH and biotinylated horseradish peroxidase H reagents (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA, USA) 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).

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

We used immunochemical staining to demonstrate infiltration of CD4+ cells in muscle. The procedures were similar to those used above to detect HA, except the primary antibody was rat anti-mouse CD4 (clone No: RM4-5, BD Biosciences Pharmingen, San Diego, CA, USA).

Enzyme-linked immunosorbent assay (ELISA) for anti-beta-galactosidase IgG

We used ELISA to determine anti-beta-galactosidase antibody in the sera of the animals. *E. coil* beta-galactosidase (Sigma, St. Louis, MO, USA) was coated onto ELISA plates (50 ng per well) and incubated at 4°C overnight. After blocking with blocking buffer at room temperature for 1 hour, serially diluted sera were added and incubated at room temperature for 1 hour. After washing with washing buffer, horseradish peroxidase-conjugated goat anti-mouse immunoglobulin antibody (Zymed, South San Francisco, CA, USA) was applied and *O*-phenylenediamine dihydrochloride TMB buffer was added for color development (Kirkegaard & Perry Laboratories, Inc. Gaithersburg, MD, USA). The reactions were detected by a microreader (MTX Lab Systems, Inc., Vienna, VA, USA) at 650 nm. Mouse sera were diluted serially from 1:64. Endpoint titers were calculated as reciprocal dilutions fourfold above background values (substrate only).

Statistical analysis

All the data were analyzed by the nonparametric Kruskal- Wallis test. Statistical significance was set at $p < 0.05$.

RESULTS

In vivo expression of *E. coli* beta-galactosidase and anti-CTLA4 scFv

We evaluated *in vivo* expression of the transgenes, beta-galactosidase and anti-CTLA4 scFv, by histochemical and immunohistochemical methods, respectively. Only a few muscle fibers expressed anti-CTLA4 scFv after repeated intramuscular injections. In addition to expression on the cell membrane, this protein was also found in the cytosol (Fig. 1). X-gal histochemical stain showed beta-galactosidase in the muscle fibers of mice in both groups. The number of blue fibers was higher in the mice receiving coadministration of p4F10-eB7 compared with their littermates as analyzed by the nonparametric Kruskal- Wallis test (Fig. 2). Hematoxyllin-eosin staining showed obvious perimyosial and endomyosial infiltration of inflammatory cells in both groups (Fig. 3A, 3B). Regeneration changes in the muscle cells were also noted. There was no significant difference in inflammatory or regeneration changes between these two groups (Fig. 3C, 3D) Interestingly, lacking coexpression of anti-CTLA4, beta-galactosidase

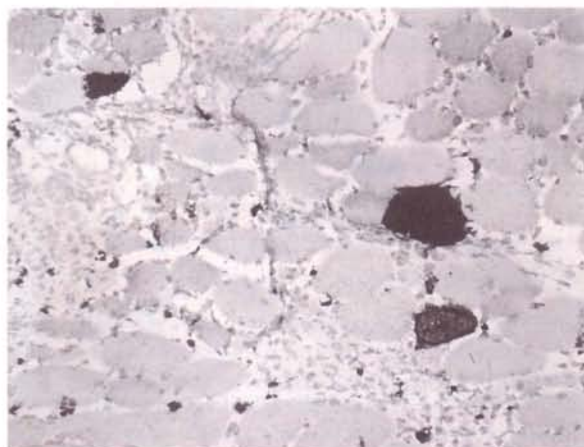

Fig. 1. *In vivo* expression of anti-CTLA4 scFv. The anti-CTLA4 scFv contains a hemagglutinin epitope near its N-terminus. *In vivo* expression of anti-CTLA4 scFv was evaluated by immunochemical staining with anti-HA monoclonal antibody. There are only a few muscle fibers expressing anti-CTLA4scFv. Although there is a transmembrane-bound domain on anti-CTLA4scFc, this protein is found in cytosol, in addition to cell membrane.

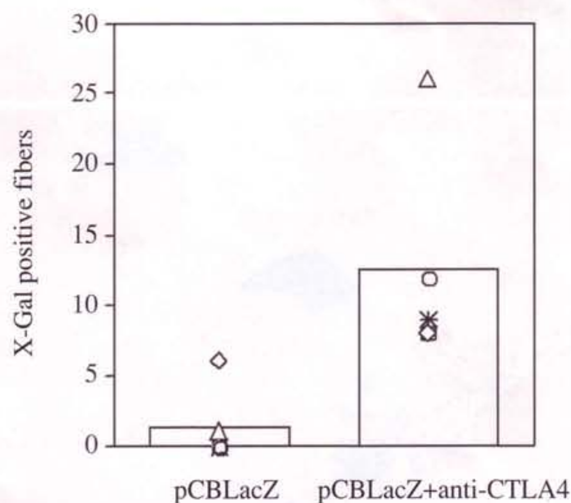

Fig. 2. *In vivo* expression of beta-galactosidase in the mice receiving coadministration of p4F10-eB7 and pCBLacZ, and in those receiving pCBLacZ alone. The animals that received two plasmids had more X-gal positive fibers after repeated intramuscular injections. (n=5, p= 0.008 by nonparametric Kruskal-Wallis test.)

could only express in the area with less inflammatory cell infiltration. However, beta-galactosidase could be found even in the areas with obvious inflammatory changes in the mice that received coadministration of p4F10-eB7. (Fig. 3E, 3F).

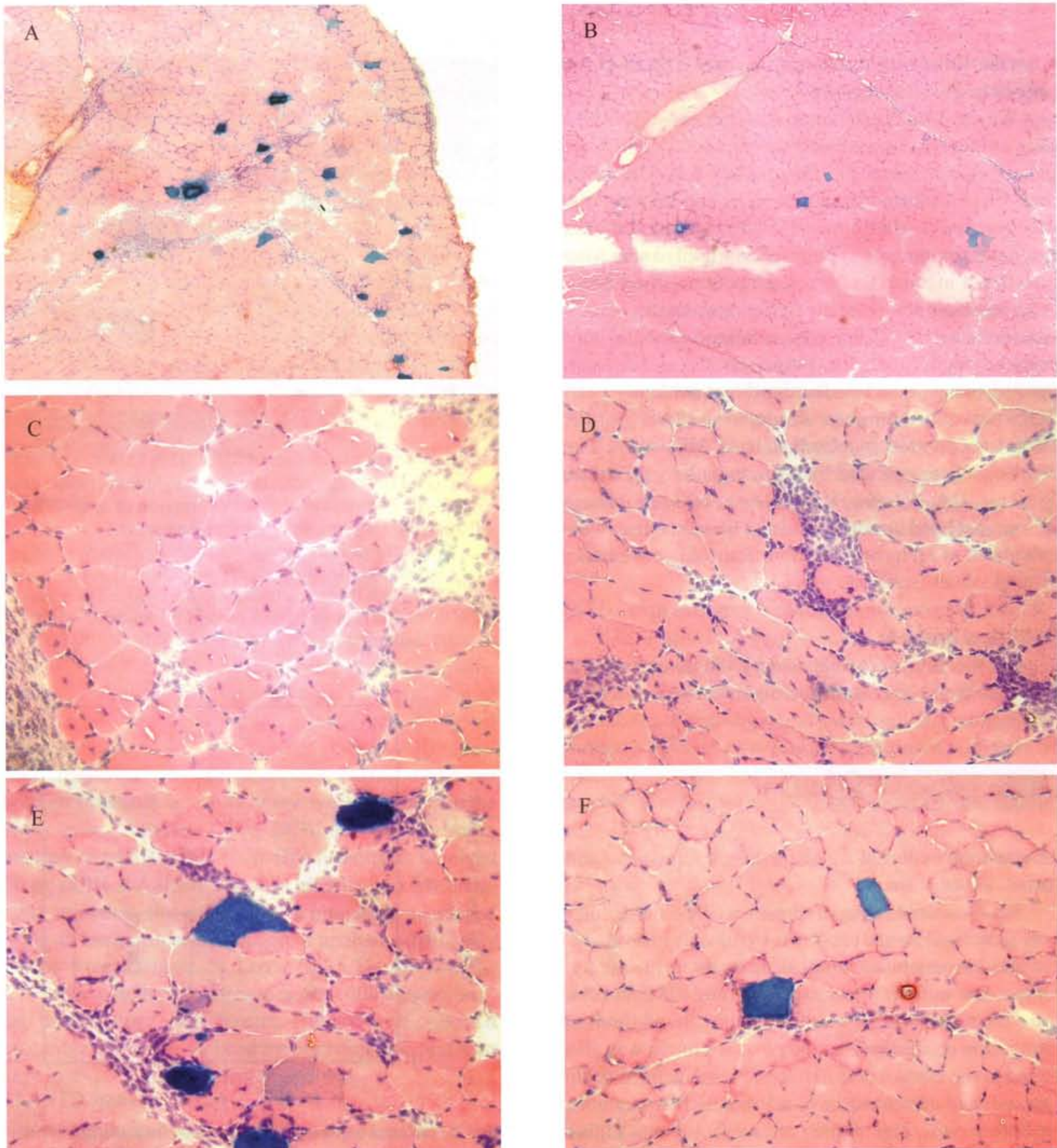

Fig. 3. Histopathological changes in muscle expressing beta-galactosidase. There are obvious endomy-osial and peiomyosial infiltrations of inflammatory cells in the animals receiving either coadministration with p4F10-eB7 (A,C) or pCBLacZ alone (B,D). (C,D) Regeneration changes, such as central nuclearization, are also found in both groups. These changes are not different between these two groups. (E) In the study group, the transduced cells are surrounded by mononuclear cells. (F) In the control group, we find that only the muscle fibers not surrounded by inflammatory cells express beta-galactosidase.

Infiltration of CD4+ cells

We analyzed CD4+ cell infiltrations by immunohistoochemical staining. Infiltration was estimated as the percentage of muscle area infiltrated by CD4+ cells under grid microscopy at 100 magnifications. There were no significant differences between these two groups by nonparametric Kruskal- Wallis test, although the filtration of CD4+ cells seemed more prevalent in some mice receiving coadministration (Fig. 4, 5). These results show that the expression of anti-CTLA4 scFv does not decrease infiltration of lymphocytes. This finding may suggest that anti-CTLA4 scFv does not attenuate host cellular immunity, so expression of beta-galactosidase may be due to local protective effects of anti-CTLA4 scFv.

Humoral immune responses

We evaluated serum anti-beta-galactosidase IgG titers in the mice receiving repeated intramuscular injections by ELISA. The endpoint titer of IgG was 1:5325 in the control group compared with 1:2253 in the study group. The p value was 0.328 by nonparametric analysis. These data suggest local expression of anti-CTLA4 scFv does not influence the host humoral immune response (Fig. 6).

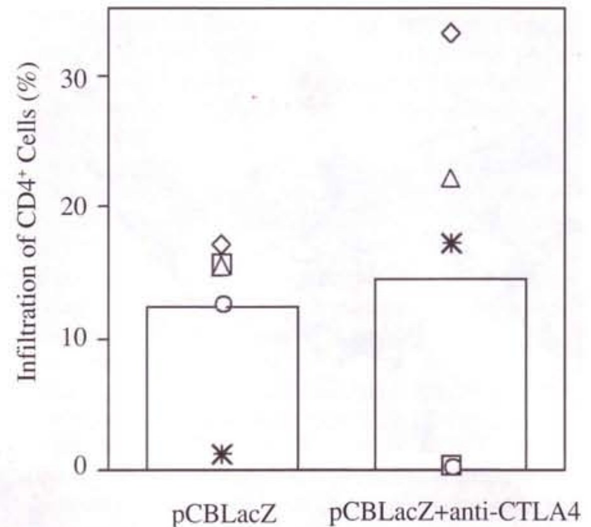

Fig. 4. Quantitative analysis of infiltration of CD4+ in treated muscle samples. The infiltration was scored by estimating the percentage of the area of the muscle slice that contained CD4+ cells under light microscopy equipped with a grid at 100 x magnification. The data are presented as mean values (standard error (n=5, p=0.60 by nonparametric Kruskal-Wallis test.)

DISCUSSION

Our study shows that coadministration of anti-CTLA4 scFv can protect transduced muscle cells from eradication by inflammatory cells in immunized animals. Although there were statistically significant differences between the study and control groups, the expression of beta-galactosidase was still much lower than that expressed in naive mice after intramuscular injection of a plasmid encoding beta-galactosidase [13]. When we evaluated the expression of anti-CTLA4 scFv, we only found a few muscle fibers expressing HA on immunochemical stain (Fig. 1). The problem may have occurred because we used CMV promoter to drive anti-CTLA4 scFv and used a hybrid promoter to encode beta-galactosidase. The latter is more potent than the former [14]. We propose that expression of beta-galactosidase was much higher than the expression of anti-CTLA4 scFv, so the small amount of anti-CTLA4 scFv was not strong enough to protect transduced cells. To overcome this problem, we could construct a dicistronic expression cassette encoding beta-galactosidase and anti-CTLA4 scFv [15]. We could also construct a plasmid containing two expression cassettes to drive these two genes with the same promoter. The problem may also be also caused by an immune response. Co-expression of this non-immunogenic or low-immunogenic single chain antibody with a strong immunogen, beta-galactosidase by antigen presenting cells (APC) could result in a bystander effect to this combination and result in an autoimmune-like response. This effect induces host immune responses against anti-CTLA4 scFv. To overcome this problem, we may try to clone these two proteins into a muscle-specific promoter.

From the hematoxyllin-eosin stain, we found numerous mononuclear cells infiltrating perimyosial and endomysial areas. Interestingly, in the mice receiving p4F10-eB7, transduced cells were found in areas with obvious inflammatory changes, but not in the control group (Fig. 3E, 3F). There was no significant difference in IgG titer between these two groups either. Because this membrane bound CTLA4 agonist acts by direct interaction with CD152 on activated T cells and causing anergy in these cells, we propose that this novel protein does not attenuate host systemic cellular or humoral immune responses, but protects the transducer at the local site.

This may suggest that anti-CTLA4 scFv does not attenuate host cellular immunity, but does protect transduced cells from elimination by host immunity.

Several methods have been tried to prolong the survival of transgenes or transplants by interrupting the costimulatory pathway [10,16,17] or transgene [18]. Jiang et al used CTLA4Ig to prolong expression of

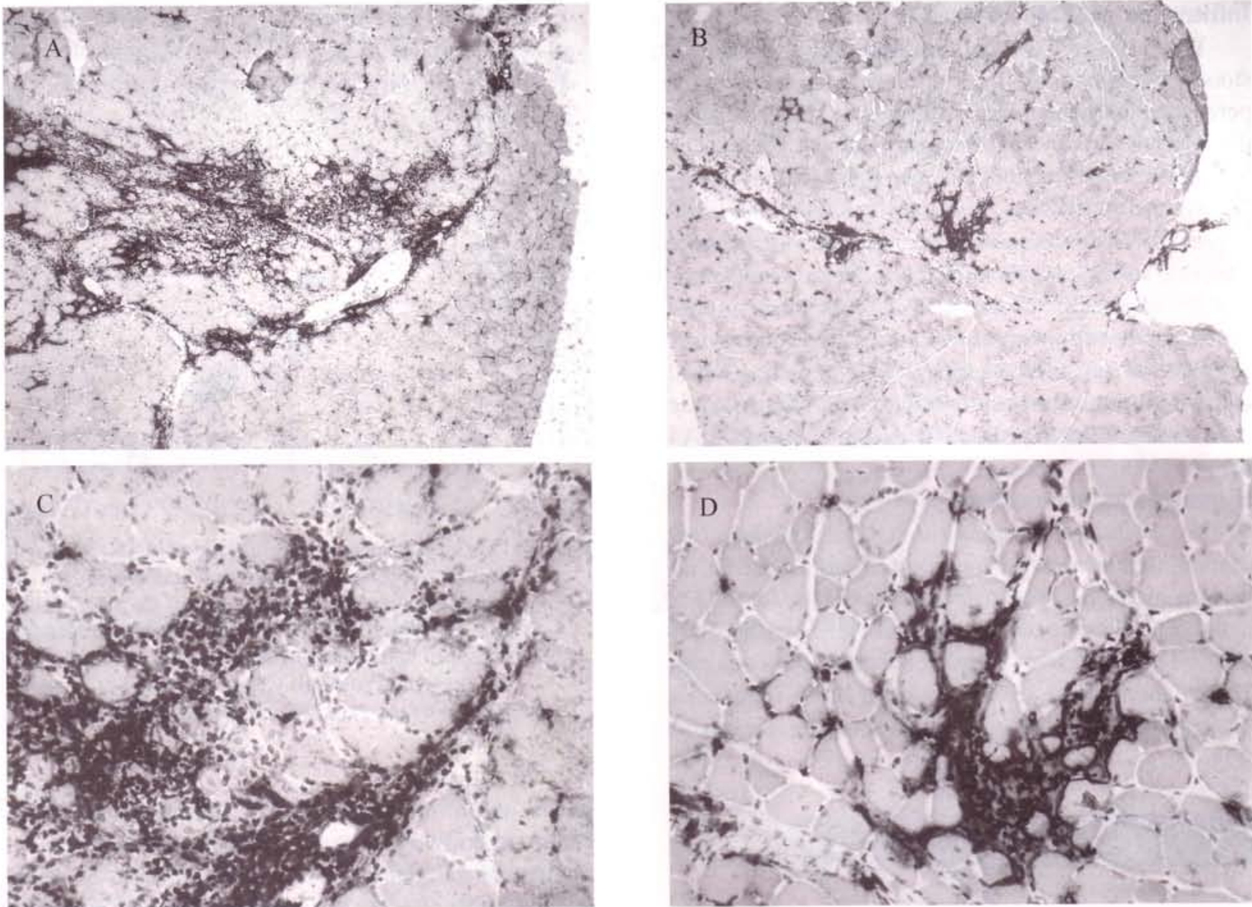

Fig. 5. The infiltrations of CD4+ cells in the mice receiving either coadministraion with p4F10-eB7 (A,C) or pCB LacZ alone (B, D). The infiltration seems more prominent in the mice receiving two plasmids. However, there are no significant differences between these two groups.

dystrophin in mdx mice after intramuscular injection of fully-gutted adenovirus. That study showed that neutralization antibody, and infiltration of lymphocytes and release of cytokines from cultured splenocytes decreased in mice receiving CTLA4Ig [19]. *In vitro* study showed that this membrane bound single chain antibody could inactivate T cells, decrease cytokine release and inhibit T cell proliferation [20]. Our data showed no significant differences between groups in cellular and humoral immunity. Unlike soluble recombinant CTLA4Ig, our single chain antibody was only expressed on the membrane of transduced cells and did not affect host immunity. The benefit of this membrane-bound single chain antibody is that it offers local protection for transduced muscle cells and does not affect host immunity.

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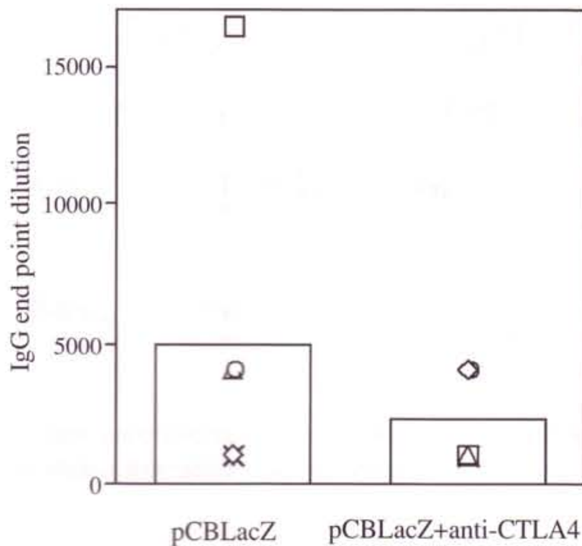

Fig. 6. Humoral immune responses against beta-galactosidase. Mouse sera were collected by cardiac puncture at the end of the study. The presence of anti-beta-galactosidase IgG was evaluated by ELISA. The concentrations of anti-beta-galactosidase IgG were presented as the end point dilutions. There is no significant difference between these two groups.

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