Cre/loxP-mediated CTLA4IgG gene transfer induces clinically relevant immunosuppression via on–off gene recombination in vivo

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Abstract

Objective: Transfer of the CTLA4IgG gene induces long-term and high levels of CTLA4IgG expression, which can result in generalized immunosuppression. In this study, we utilized Cre/loxP-mediated on–off switch recombination to eliminate transgene expression of CTLA4IgG following acceptance of murine cardiac allografts.

Methods: Fully MHC-mismatched hearts from BALB/c donor mice were transplanted into C3H/He recipient mice. Adenovirus-containing CTLA4IgG flanked between two loxP sites was administered via a recipient tail vein immediately after transplantation. Cre-recombinase gene was subsequently transferred at day 30 posttransplantation.

Results: Long-term allograft survival was observed in recipients that received the CTLA4IgG gene. Cre-mediated recombination reduced CTLA4IgG gene expression without any adverse effect on the graft survival. Secondary skin grafts of donor type and of third party were promptly rejected in the recipients that accepted cardiac allografts. In addition, the B cell response against ovalbumin was suppressed during high levels of serum CTLA4IgG, but recovered after Cre-mediated inactivation of CTLA4IgG gene.

Conclusion: CTLA4IgG gene transfer promoted long-term survival of murine cardiac allografts; however, this was not sufficient to induce tolerance. Cre/loxP-mediated on–off switch recombination was useful to inactivate the CTLA4IgG gene so that recipients’ immune responses against neoantigens were restored without an influence on the allograft survival. This system may open novel strategies to orchestrate clinically relevant immunosuppression.

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Keywords: CTLA4IgG; Transplantation; Gene therapy; Allograft rejection; Cre/loxP recombination

1. Introduction

Cardiac transplantation has evolved into a standard treatment for selected patients with end-stage heart failure. Various immunosuppressive strategies have been investigated and reported to reduce graft rejection; however, there is an extensive need for the development of antigen specific tolerance without generalized immunosuppression.

It has been known that T cells require two distinct signals for full activation. The first signal is the T cell receptor-mediated signal, which is provided with the MHC/antigenic peptide complex. The second signal is delivered by costimulatory molecules interacting between specific receptors on the T cell and their respective ligands on antigen-presenting cells. The prototypical costimulatory signal between CD28 on T cells and B7.1 or B7.2 on activated...
antigen-presenting cells is essential for initiating T cell proliferation and differentiation [1–4]. The use of costimulatory blockade inhibits T cell activation and is marvelously effective in prolonging allograft survival in numerous models of cell and solid-organ transplantation [5,6], as well as in treatment of autoimmune diseases [7,8]. CTLA4IgG, a recombinant fusion protein that contains the extracellular domain of mouse CTLA4 and the Fc portion of human IgG1, competitively inhibits the binding of B7 molecules to CD28. Blockade of the CD28-B7 pathway by systemic administration of soluble CTLA4IgG proteins has been one of the accepted immunosuppressive strategies in allotransplantation, and clinical trials are now under way in kidney transplant recipients [1–4,9–14].

Transfer of an adenovirus vector containing CTLA4IgG gene has been previously reported to prolong allograft survival of a number of experimental models [15–21]. The transgene expression of CTLA4IgG; however, has been achieved over a period of several months in that excessive immunosuppression against exogenous antigens can be predictable. The Cre/loxP system utilizes Cre recombinase to catalyze excision of DNA located between flanking two loxP sites and is a useful strategy for conditional gene expression or inactivation [22–25]. A new adenoviral vector: Adex1CALoxCTLA4IgGLox contains survival of a number of experimental models [15–21]. The care and use of Laboratory Animals published by the US Institute of Laboratory Animals at the Shinshu University School of Medicine.

2. Materials and methods

All experimental procedures conform to the Guide for the care and use of Laboratory Animals published by the US National Institute of Health.

2.1. Animals

Male BALB/c (H-2b) mice (age 4–6 weeks, 20–25 g) and male C3H/He (H-2k) mice (age 4–6 weeks, 20–25 g) were obtained from Japan SLC, Inc., Shizuoka, Japan. All mice were housed in pathogen-free conditions in our Institute of Laboratory Animals at the Shinshu University School of Medicine.

2.2. Recombinant adenoviruses

Adex1CALoxCTLA4IgGLox was prepared as follows: a cDNA fragment encoding CTLA4IgG was excised from the expression vector CDM8-CTLA4IgG [27] and cloned between the loxP sequences under the control of the CAG promoter in pCALwL [28], and then an expression unit, CAG promoter-loxP-CTLA4IgG-loxP-poly(A), was isolated. The resultant expression unit was cloned into the cosmid vector pAdex1CA, which contained an entire adenovirus type 5 genome except for the E1 and E3 regions (pAdex1CALoxCTLA4IgGLox). Recombinant adenoviruses were constructed by in vitro homologous recombination in 293 cells by using pAdex1CALoxCTLA4IgGLox and the adenovirus DNA terminal-protein complex. The desired recombinant adenoviruses, termed Adex1CALoxCTLA4IgGLox, and Adex1CACre consisting of the Cre gene under the control of the CAG promoter, were propagated in 293 cells and purified by two rounds of cesium chloride density centrifugation as described previously [29,30]. The concentrated viruses were dialyzed against phosphate-buffered saline – 10% glycerol. Adenovirus vector having β-galactosidase (Adex1CALacZ) was used as a control vector. The titer of the virus stock was assessed by a plaque formation assay that used 293 cells.

2.3. Transplantation and adenovirus-mediated gene transfer

Abdominal heterotopic vascularized cardiac transplantation was performed using microsurgical techniques as previously described [31]. Mice were anesthetized with intraperitoneal 4% chloral hydrate (0.01 mL/g). Fully MHC-mismatched hearts from BALB/c donors were transplanted as allografts into C3H/He recipients. The survival of cardiac allografts was assessed by daily palpation. Secondary skin grafts of both donor (BALB/c) and third-party (C57BL/6) origin were transplanted simultaneously onto recipients who accepted cardiac allografts for more than 120 days. All adenoviral vectors were administered via tail vein. Transplantation and treatment protocol consisted of 7 experimental groups of mice as follows (Table 1): Control mice received cardiac allografts and left untreated (group I) or treated with 5 × 10⁷ of control vector Adex1CALacZ alone on day 0, immediately after transplantation (group II). Mice in groups III, IV, V, and VI received cardiac allografts and 5 × 10⁷ of Adex1CALoxCTLA4IgGLox on day 0. Group III was then left untreated. Groups IV and V were subsequently treated with 1 × 10⁹ or 2 × 10⁹ of Adex1CACre on post-operative day 30; thus the multiplicity of infection (MOI) was 20 and 40, respectively. Group VI was followed by Adex1CALacZ administration on day 30 as control. While mice in group VII received 5 × 10⁷ of Adex1CALoxCTLA4IgGLox on day −60, 2 × 10⁹ of Adex1CACre on day −30, and were performed cardiac allografts on day 0, when we confirmed elimination of serum CTLA4IgG expression.

2.4. Histologic analyses

Cardiac allografts were harvested 14 days after transplantation. Removed allografts were fixed in 10% buffered formalin and embedded in paraffin. Ventricular short-axis sections were cut and stained with hematoxylin and eosin.
moi: multiplicity of infection, PFU: plaque-forming unit.

(H&E) to assess allograft rejection. Ten days after intravenous Adex1CALacZ administration, enzymatic β-galactosidase staining was performed to detect LacZ expression on each tissue section of the recipient liver, the lungs, the spleen, the brain, the kidneys, the intestine, and the heart, as well as cardiac allografts.

2.5. Immunohistochemistry

Paraffin-embedded sections were deparaffinized and rehydrated. Since CTLA4IgG consists of murine CTLA4 and the Fc portion of human IgG1, tissue sections were stained with polyclonal goat antimouse CTLA4 (sc-1630, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 1:500 dilution, Santa Cruz Biotechnology, Inc., Santa Cruz, CA. Enzyme activity was detected with dianinobenzidine and sections were counterstained with Mayer’s hematoxylin.

2.6. In situ hybridization

Total RNA was extracted from liver tissue 3 days after CTLA4IgG gene delivery, cDNA was synthesized by RT, and PCR was performed using a pair of primers according to the genomic sequences in the regions of exon 2 of mouse CTLA4 gene (AF142145, GenBank). The 120-bp PCR products were sub-cloned into pCR™II vector (Invitrogen Co., Carlsbad, CA). Sense and anti-sense RNA probes were transcribed from the templates with Sp6 (sense) and T7 (anti-sense) using DIG RNA Labeling Kit (Roche Co., Basel, Switzerland). After the tissue sections were deparaffinized in xylene, hydrated slides were immersed in 0.2 mol/L HCl for 20 min and then digested with 100 μg/ml proteinase K at 37 °C for 20 min, followed by postfixation with 4% paraformaldehyde. These slides were rinsed with 2 mg/ml glycine and then acetylated for 10 min in freshly prepared 0.25% acetic anhydride in 0.1 mol/L triethanolamine (pH 8.0). The hydrated slides were deffatted with chloroform and then air-dried. After prehybridization with 50% deionized formamide/2× standard saline citrate (SSC) for 60 min at 45 °C, the slides were hybridized with 0.5 mg/ml of the antisense or sense probe in 50% deionized formamide, 2.5 mmol/L EDTA (pH 8.0), 0.3 mol/L NaCl, 1× Denhardt’s solution, 10% dextran sulfate, and 1 mg/ml brewer’s yeast tRNA at 45 °C for 16 h. After hybridization, the slides were washed in 50% formamide/2× SSC for 60 min at 45°C and digested with 10 mg/ml RNase A at 37 °C for 30 min. After washing sequentially with 2× SSC/50% formamide at 45 °C for 60 min, 1× SSC/50% formamide at 45 °C for 30 min, and 1× SSC/50% formamide at room temperature for 30 min, the sections were incubated with alkaline phosphatase-conjugated anti-digoxigenin IgG and detected with the chromogenic agents nitro blue tetratholium and 5-bromo-4-chloro-3-indolyl phosphate.

2.7. Detection of serum CTLA4IgG

Serum concentrations of mouse CTLA4IgG were assayed by the enzyme-linked immunosorbent assay (ELISA). Sample sera were collected periodically from the tail vein of all mice in each group (Table 1). Goat anti-human IgG (Jackson Immunoresearch Laboratories, Bar Harbor, ME) was coated onto 96-well plates. Diluted serum samples or standard CTLA4IgG was added to the blocked wells. After washing, hamster anti-mouse CTLA4 antibody, 4F10 (a kind gift from J. Bluestone, Chicago University), was added followed by peroxidase-labeled goat anti-hamster antibody (Jackson ImmunoResearch Laboratories). Finally, the substrate o-phenylenediamine-H2O2 was added and absorbance at 490 nm was measured. Each sample was analyzed in duplicate.

2.8. Anti-ovalbumin antibody titer

Untreated control mice and allograft recipients were subcutaneously immunized with 100 μg of ovalbumin (OVA, fraction V; Sigma-Aldrich Co., St. Louis, MO) in 100 μl of Complete Freund’s Adjuvant to evaluate B cell immune response against exogenous antigen. Anti-OVA antibody titers in serum samples were determined by ELISA, as previously described [32,33]. In brief, 96-well plates were precoated with OVA (10 μg/ml) and subse-
sequently blocked with 1% BSA for 2 h at room temperature. Serum samples (100 μl) were then applied to plates at 4 °C overnight. After washing, peroxidase-conjugated anti-mouse IgG was added and incubated for an hour. Finally, the color reaction was developed with TMB Peroxidase Substrate System (50-76-00, Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD), and the plates were read at 450 nm. All serum samples were duplicated at 1:80000 dilution and absorbance values for the duplicate wells were averaged.

2.9. Statistical analyses

All data are expressed as mean±SEM. To analyze allograft survival, the log-rank comparison of the groups was used to calculate P values. Statistical differences between groups in the ELISA assay were determined using Mann--Whitney U test. Values of P<0.05 were considered statistically significant.

3. Results

3.1. β-galactosidase staining

To evaluate tissue distribution of systemic gene transfer using the recombinant adenovirus vectors, β-galactosidase staining was performed 10 days after Adex1CALacZ delivery. β-galactosidase-positive cells were detected predominantly in the cytoplasm of the recipient hepatocytes (Fig. 1A), but also in the cardiac allografts (Fig. 1B). Minimal staining was also found in the recipient lungs and the spleen.

3.2. CTLA4IgG expression on tissue sections

Four days after transfer of Adex1CALoxCTLA4IgGLox, CTLA4IgG expression was evaluated on tissue sections of allograft recipients. Positive staining was detected in the recipient liver using both immunohistochemistry (Fig. 2A) and in situ hybridization assays (Fig. 2B), which presented protein and RNA expressing cells, respectively. The cytoplasms of periportal hepatocytes were similarly stained by each assay. This suggests that the liver was the target organ of adenoviral mediated CTLA4IgG gene delivery in this study, and that recipient hepatocytes were the source of serum CTLA4IgG protein. Whereas no positive staining was found on either tissue section from the lungs, the spleen, the brain, the kidneys, the intestine, the recipient heart, or cardiac allografts. Following Adex1CACre-mediated gene recombination, CTLA4IgG was not expressed in any of these sections, as demonstrated by both immunohistochemistry and in situ hybridization assays.

3.3. Serum CTLA4IgG concentration

Serum concentrations of CTLA4IgG in the allograft recipients were still sustained at as much as 60 μg/ml on 84 days after the single administration of Adex1CALoxCTLA4IgGLox on the day of transplantation (group III). Following administrations of Adex1CACre at 30 days posttransplantation (groups IV and V), the serum CTLA4IgG levels were significantly suppressed as compared with groups III and VI (Fig. 3). Statistical differences were found on days 42, 49, and 56 (P<0.05 on each day), which were 12, 19, and 26 days after Adex1CACre administration, respectively. This highlights the in vivo manipulation of serum CTLA4IgG protein production, and suggests the function of Adex1CACre-mediated on–off gene recombination.

3.4. Graft survival

Untreated control recipients (group I) or recipients treated with Adex1CALacZ alone (group II) promptly rejected cardiac allografts between 8 and 12 days (Table

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Fig. 1. Function of adenovirus-mediated gene transfer following intravenous administration via recipient tail vein. β-galactosidase positive cells (blue) were detected in tissue sections of the recipient liver (A) and of the cardiac allograft (B), which were harvested from C3H/He recipients at 10 days after Adex1CALacZ delivery. Bars: 50 μm.
1). **CTLA4IgG** gene delivery using Adex1CALoxCTLA4Ig-GLox promoted long-term allograft survival (group III; \( P < 0.01 \) as compared with either group I or II). It was remarkable that no significant decrease in the graft survival was found following Adex1CACre-mediated gene recombination (groups IV and V; \( P \) = not statistically significant for either, as compared with group III). We next transferred Adex1CALacZ (group VI), as a control vector for Adex1CACre, and found no additional effect on either allograft survival (\( P \) = not statistically significant) or the manipulation of serum CTLA4IgG expression (Fig. 3). Moreover, when cardiac allografts were transplanted after the **CTLA4IgG** gene ablation (group VII), all grafts were rejected at a rate similar to that of control recipients in groups I, and II (\( P \) = not statistically significant). This suggests that **CTLA4IgG** gene had been inactivated prior to the cardiac transplantation. In fact, the serum concentrations of CTLA4IgG in group VII were below measurable levels at a few days pretransplantation. To evaluate antigen-specific unresponsiveness, secondary skin grafts were transplanted onto recipients who had accepted C3H/He hearts over 120 days posttransplantation, when we confirmed the termination of serum CTLA4IgG expression (groups IV and V). Recipients either with (groups IV and V) or without Cre-mediated recombination (group III) promptly rejected skin grafts of both donor origin (BALB/c) and third-party (C57BL/6) within 16 days. Tolerance to skin grafts, therefore, was not induced under circumstances of high levels of serum **CTLA4IgG**.

### 3.5. Microscopic findings of cardiac allografts with H&E stain

Tissue sections of cardiac allografts were obtained from recipients on day 14 posttransplantation. Rejected allografts from untreated control mice (group I) and from Adex1CALacZ treated mice (group II) similarly demonstrated significant inflammatory cell infiltration and interstitial hemorrhage (Fig. 4A and B). **CTLA4IgG** gene transfer (group III) markedly prevented the acute rejection (Fig. 4C), in which sparse interstitial infiltrate of mononuclear cells was found, but microvessels and the perivascular area remained intact. These histological findings mirrored the survival data.

### 3.6. Immune responses against OVA

To further test B cell responses against exogenous antigens, we analyzed anti-OVA antibody production against OVA immunization. When untreated control mice were immunized with OVA on day 0 (\( n = 5 \)), significant production of anti-OVA antibody was detected over 50 days
after the single immunization (Fig. 5, ●). Next, we immunized recipients of functioning cardiac allografts with the following protocol: C3H/He mice received BALB/c hearts at 60 days before immunization (day – 60) along with Adex1CALoxCTLA4IgGLox transfer immediately after transplantation. These were followed by Adex1CACre administration on day – 30. Five recipients were immunized with OVA twice: the first was on day – 56 under high levels of serum CTLA4IgG, and the second was on day 0 when we confirmed CTLA4IgG termination (Fig. 5, ▼). Another five recipients received single immunization on day 0 (Fig. 5, ▲). The results were as follows: First, recipients without primary immunization produced anti-OVA antibodies at levels similar to that of untreated control mice. Second, the antibody production against primary immunization was completely suppressed from day – 56 to day – 1 in recipients with sufficient levels of serum CTLA4IgG (Fig. 5, ▼). This indicates potent inhibitory effect of CTLA4IgG on B cell immune responses to the neoantigen. Finally, following the primary immunization and subsequent CTLA4IgG elimination, reduced antibody production against the second OVA immunization was seen on days 42, 49 and 56, as compared with the control group (P < 0.05 on each day); however, it was still found at about half the absorbance values as the controls (Fig. 5, ▼). These findings suggest that after Cre-mediated CTLA4IgG gene recombination, recipient mice with functioning allografts were partially able to mount antibody production against exogenous antigens, and that recipient B cells were not tolerated under high levels of serum CTLA4IgG.

4. Discussion

Recombinant adenoviral vectors have been one of the most efficient gene transfer systems, and in vivo transfer of the CTLA4IgG gene significantly prolongs cardiac allograft survival [15,16]. Our previous reports also demonstrated

![Fig. 5. B cell immune response against OVA immunization during the on–off manipulation of CTLA4IgG gene expression. Three protocols of treatment and immunization were described in the table (bottom). Serum levels of anti-OVA antibody were periodically assessed by ELISA and mean ± SEM values were derived from 5 mice in each group. Control mice demonstrated significant production of anti-OVA antibodies over 50 days after immunization (●). Following single immunization at 30 days after CTLA4IgG recombination, recipient mice produced the antibodies (▲) at the same levels as that of control mice. Note that the B cell immune response was completely suppressed during the period of high levels of serum CTLA4IgG from day – 56 to day – 1 (▼); furthermore, decreased response was found following CTLA4IgG gene recombination in the recipients primarily immunized on day – 56 (*P < 0.05).](https://academic.oup.com/cardiovascres/article-abstract/69/1/289/348266)
potent efficacy of Adex1CACTLA4IgG transfer in a long-term acceptance of allografted islet cells [26], in treatment of murine type II collagen-induced arthritis [34], and in prevention of experimental autoimmune myocarditis [35]. The high and long-lasting transgene expression of CTLA4IgG, however, has the potential to cause extensive immunosuppression against exogenous antigens. In general, the host immune responses against adenoviral proteins may preclude or reduce the efficacy of vector administration in transfected tissues [36,37], whereas the constitutive expression of CTLA4IgG may attenuate immune responses and prolong transgene expression [30,38]. In fact, adenovirus-mediated CTLA4IgG expression lasts for several months and completely prevents the production of antibodies against adenovirus [30] and donor alloantigens [16]. In this study, we focused on the function of Cre/loxP-mediated on–off gene recombination in manipulating the adenovirus-mediated CTLA4IgG expression and thereby preventing generalized immunosuppression.

Progress in manipulating DNA molecules utilizing the Cre/loxP gene targeting system has been fully appreciated. Cre-recombinase catalyzes the excision of DNA segment flanked by two loxP sites with no requirements for accessory proteins or specific substrates [22,23]. Temporal, spatial, and cell type-specific control of Cre-mediated DNA recombination has been reported [24]. In particular, cardiac myocyte-specific [39] or endothelium-specific [40] recombination has been demonstrated using Cre cDNA, which is controlled by either cardiac-specific α-myosin heavy chain promoter or endothelial cell lineage-specific Tie2 promoter, respectively. Moreover, utilizing the cardiac-specific gene recombination system, the function of GATA4 gene [41] and serum response factor gene [39] required for cardiac development have recently been revealed. We constructed adenovirus vectors containing either CTLA4IgG cDNA between flanking loxP sequences (Adex1CALoxCTLA4IgGLOx), or Cre-recombinase gene (Adex1CACre), which was subsequently expected to terminate CTLA4IgG expression in vivo. While the recombination was arbitrarily assignable, it was performed 30 days after transplantation because CD28 signal blockade by CTLA4IgG plays a major role during the priming phase of alloimmune response. We demonstrated here that Cre/loxP-mediated recombination trimmed serum levels of CTLA4IgG expression so that it appeared similar to a rectangular curve. This on–off switching system, therefore, could be expected to minimize the risk of deleterious immunosuppression and also to improve the efficacy and safety of adenovirus-mediated CTLA4IgG gene transfer.

Since intravenous gene deliveries using adenovirus vectors have been predominantly transferred into the liver [36], it was expected that β-galactosidase positive cells and CTLA4IgG expression were detected mainly in the cytoplasm of hepatocytes. The systemic gene delivery, however, may possibly transfer target genes to any of recipient organs. In fact, β-galactosidase staining was also found in the cardiac allograft. Following the Adex1CACre administration, CTLA4IgG was not detected either by immunohistochemistry or by in situ hybridization assays (data not shown), accompanied with the significant decrease in levels of serum CTLA4IgG. This suggests that adenoviral gene delivery and subsequent Cre-mediated on–off recombination occurred in the liver, and that the positive hepatocytes were the source of circulating CTLA4IgG. These findings are consistent with recent reports describing the liver as an ideal target for gene therapy [36,42].

Safety and feasibility aspects of the adenovirus-mediated gene transfer into the liver are worth discussing. A previous Phase II clinical trial for patients with gastrointestinal carcinoma metastatic to the liver has demonstrated that repeated adenoviral infusions via hepatic artery are well tolerated with minimal toxicity [43], and a randomized Phase III trial utilizing a replication-selective oncolytic adenovirus is underway. In contrast, no clinical applications of gene therapy in heart transplantation have been reported. In addition to the safety of the adenoviral infusion into the bloodstream, the Cre/loxP-mediated on–off gene recombination system particularly needs to be certified that almost all of the liver cells which receive Adex1CALoxCTLA4IgGLOx also receive Adex1CACre in a second independent transfection. Thus, similar transfection efficiency and distribution of the adenovirus vectors in the liver appear to be essential for this system to move forward in human transplantation medicine.

Recent reports [12–14] and the present study demonstrated long-term survival of murine cardiac allografts treated with CTLA4IgG alone; however, moderate but not long-term survival prolongation have been reported in rat allograft models [9,15]. These inconsistencies in results are probably because of the variability in mechanisms of innate immunity among species and differences between treatment protocols. Further investigation is required to evaluate the effect of CTLA4IgG in human recipients of cardiac allografts.

Although induction of donor-specific tolerance has been reported utilizing adenovirus coding CTLA4IgG in some experimental transplantation models [16,20,21], skin grafts from both donor type and 3rd-party mouse were rejected within 16 days on recipients in either group III, group IV, or group V, which had accepted cardiac allografts. Furthermore, B cell tolerance was not induced against OVA-immunization under the circumstance of high CTLA4IgG concentration. These results are in keeping with recent findings that CD28-B7.1/B7.2 signal blockade via CTLA4IgG leads to long-term allograft survival, but the immune regulation by itself is not sufficient to induce long-lasting robust tolerance [7,9,17–19,44]. Since secondary cardiac allografts were not performed in this study, there is a limitation in that we could not evaluate organ-specific tolerance. Nevertheless, tolerance induction via CTLA4IgG treatment alone remains still controversial as suggested by the following: Mechanisms of costimulation-resistant rejec-
tion are reported in CD28/CD154 double-knockout mice [45], as well as in mice receiving combined CD28/CD40 blockade [46]. In fact, complete blockade of CD28 signaling via CTLA4IgG may abrogate a beneficial negative regulatory signal through CTLA4 [11,14], which regulates induction of a division-arrest-associated form of T cell anergy in vivo [47,48]. In fact, combined AdexCTLA4Ig and AdexCD40Ig gene transfer allowed long-term acceptance of cardiac allografts; however, this regimen was not sufficient to induce tolerance to skin grafts and to prevent chronic cardiac allograft rejection [49]. Concomitant use of costimulatory blockades with strategies involving donor-specific transfusion, donor dendritic cells, or mixed chimerism may be required to induce tolerance to cardiac allografts [9,10,50].

In this study, we established that Cre/loxP-mediated on–off gene recombination eliminated in vivo transgene expression of CTLA4IgG without any adverse effect on the long-term cardiac allograft survival. This system is useful to manipulate serum or tissue expression of a number of costimulatory blockades or other therapeutic molecules, and may enable us to orchestrate clinically relevant immunosuppression. Targeting gene transfer utilizing the mechanism of time- and/or tissue-specific expression will open novel strategies to prevent cardiac allograft rejection.

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