Tolerance to Cardiac Allografts Via Local and Systemic Mechanisms After Adenovirus-Mediated CTLA4Ig Expression[1]

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Blockade of the CD28/B7 T cell costimulatory pathway prolongs allograft survival and induces tolerance in some animal models. We analyzed the efficacy of a CTLA4Ig-expressing adenovirus in preventing cardiac allorejection in rats, the mechanisms underlying heart transplant acceptance, and whether the effects of CTLA4Ig were restricted to the graft microenvironment or were systemic. CTLA4Ig gene transfer into the myocardium allowed indefinite graft survival (>100 days vs 9 ± 1 days for controls) in 90% of cases, whereas CTLA4Ig protein injected systemically only prolonged cardiac allograft survival (by up to 22 days). CTLA4Ig could be detected in the graft and in the serum for at least 1 year after gene transfer. CTLA4Ig gene transfer induced local intragraft immunomodulation at day 5 after transplantation, as shown by decreased expression of the IL-2R and MHC II Ags; decreased levels of mRNA encoding for IFN-γ, inducible NO synthase, and TGF-β; and inhibited proliferative responses of graft-infiltrating cells. Systemic immune responses were also down-modulated, as shown by the suppression of Ab production against donor alloantigens and cognate Ags, up to at least 120 days after gene transfer. Alloantigenic and mitogenic proliferative responses of graft-infiltrating cells and total splenocytes were inhibited and were not reversed by IL-2. In contrast, lymph node cells and T cells purified from splenocytes showed normal proliferation. Recipients of long-term grafts treated with adenovirus coding for CTLA4Ig showed organ and donor-specific tolerance. These data show that expression of CTLA4Ig was high and long lasting after adenovirus-mediated gene transfer. This expression resulted in down-modulation of responses against cognate Ags, efficient suppression of local and systemic allograft immune responses, and ultimate induction of donor-specific tolerance. ***The Journal of Immunology,*** **2000, 164: 5258–5268.**

Graft rejection depends on the activation of alloreactive T cells. Optimal and sustained activation of T cells, leading to proliferation, cytokine production, and effector functions, requires three signals. The first signal is delivered by the TCR after interaction with antigenic peptides presented on MHC molecules of APCs. The second, or costimulatory signal, is provided through the interaction of T cell molecules; among them, CD28 is one of the most important ones, with the APC molecules CD80 and CD86. The third signal allows amplification of the immune response and is provided by cytokine receptors after interaction with their ligands (IL-2, IL-4). Other signaling membrane molecules, among them CTLA4, transduce inhibitory signals to activated T cells (1). CTLA-4 is highly homologous to CD28, but has a higher affinity for CD80 and CD86 as compared with CD28 and also a slower kinetics of expression.

Administration of CTLA4Ig, a recombinant fusion protein that contains the extracellular domain of CTLA4 fused to IgG heavy chain constant domain, competitively inhibits binding of B7 molecules to CD28, but also to CTLA4 (1). The net effect of CTLA4Ig is to inhibit T cell activation, and thus effectively suppress immune responses in various transplantation and autoimmune models.

CTLA4Ig differs in its efficacy to prevent acute rejection or to induce transplantation tolerance (defined as permanent graft acceptance in the absence of immunosuppressors and chronic rejection) of various vascularized grafts in rodents. In rats and several mouse models, CTLA4Ig prevents acute cardiac rejection, but does not induce tolerance, even after prolonged administration, unless associated with other treatments (1) (2–5). In contrast, other vascularized grafts, such as kidney allografts, have been shown to be permanently accepted after a single administration of CTLA4Ig (5, 6).

Gene transfer of sequences coding for soluble immunosuppressive molecules into transplanted organs aims to create a local microenvironment directly modulating the activation state of immune cells responsible for graft rejection (7, 8). Therefore, when compared with systemic administration, local and continuous production of biologically active compounds might increase their bioavailability and allow a more effective treatment. Gene transfer could also allow a reduction in the costs associated with multiple drug administrations. Furthermore, cells not involved in the rejection process could be spared, and side effects or generalized immunosuppression may thus be avoided. Nevertheless, the potential benefits and drawbacks of gene transfer in transplantation, such as the localized vs systemic immunosuppressive effects, have not been rigorously analyzed.

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Gene transfer in transplantation has been performed using a variety of vectors, including recombinant adenoviruses (Ad).[3] Ad have attractive properties for transducing vascularized organs (8). We have previously shown that Ad-mediated gene transfer of TGF-β (9) or IL-10 (10) delays rejection of cardiac allografts.

Adenovirus-mediated gene transfer of murine CTLA4Ig to the rat liver (11) and cardiac (12) transplantation models resulted in prolongation of allograft survival. Nevertheless, the immune responses of grafted recipients toward nominal Ags other than alloantigens or the mechanisms underlying graft acceptance have not been fully characterized.

The aim of this study was to evaluate the efficacy of an Ad coding for murine CTLA4Ig in preventing allograft rejection in a rat cardiac allotransplantation model, to analyze the mechanisms implicated in graft acceptance by CTLA4Ig, to define whether gene transfer into the heart resulted in graft-restricted or systemic immunosuppression, and whether imunosuppression was allospecific or aspecific.

Adenovirus-mediated gene transfer of CTLA4Ig resulted in permanent graft acceptance and prolonged expression of CTLA4Ig, whereas repeated administration of recombinant CTLA4Ig (rCTLA4Ig) only moderately prolonged graft survival. Recipients of AdCTLA4Ig-treated cardiac grafts showed systemic inhibition of humoral and of cell-mediated immune responses against donor Ags (splenocytes but not of lymph node cells) and cognate Ags. Leukocytes infiltrating grafts injected with AdCTLA4Ig showed decreased expression of MHC class II Ags and CD25; reduced IFN-γ, TGF-β, and iNOS mRNA accumulation; and decreased proliferative responses to alloantigens. Recipients with permanently accepted AdCTLA4Ig-treated cardiac grafts accepted donor-matched second heart grafts, but rejected donor-matched skin and third party skin and hearts.

These results demonstrate that intragraft gene transfer of CTLA4Ig, a simple and perfectly tolerated procedure, resulted in very efficient induction of permanent cardiac graft acceptance. This effect was dependent on local and systemic immunosuppresive effects leading to the establishment of active donor- and organ-specific tolerance mechanisms.

Materials and Methods

Recombinant adenoviruses

Ad were constructed, propagated, purified, and titered (in PFU) according to standard protocols (13), as previously described (14, 15). The cDNA sequences from the extracellular portion of mouse CTLA4 and the coding sequences of the constant domains of human IgG1 (16) (kindly provided by P. Lane) were placed under the transcriptional control of a short truncated CMV promoter. Addl324 is a noncoding Ad. Adenovirus stocks were tested for the absence of replication-competent adenoviruses by PCR amplification of the E1 adenoviral region (the detection limit was 1 adenoviral particle in 10^9 PFU of Ad).

Animals, transplantation, adenovirus-mediated gene transfer, and administration of rCTLA4Ig

The rats used in this study were inbred male Lewis 1W (LEW.1W, haplotype $RT1^u$), LEW.1A (haplotype $RT1^a$), Brown Norway (BN, haplotype $RT1^n$) (Centre d'Elevage R. Janvier, Le Genest St. Isle, France), and Fischer (haplotype $RT1^{lv1}$) (IFFA CREDO, L'Arbresle, France). These are congeneic animals completely mismatched for the class I, II, and I-like genes of the MHC region. Heterotopic cardiac allografts were placed into the abdomen (first grafts) or into the neck (second grafts). Immediately after transplantation, Ad (at the indicated doses in 250 μl) were slowly injected into the apex and ventricular walls of the clamped heart at four different points (9). Graft survival was monitored daily by palpation through the abdominal wall. Rejection was defined as total cessation of cardiac beating and was confirmed by direct examination following laparotomy. Full-thickness dorsal skin from syngeneic, first, and third party donors were transplanted onto the dorsal trunk area, and skin rejection was defined as >75% graft necrosis.

The fusion protein CTLA4Ig, composed of the extracellular portion of mouse CTLA4 and the constant domains of mouse IgG1 (kindly provided by Dr. R. Peach, Bristol-Myers Squibb, Seattle, WA), was administered daily (i.p., 50 μg) from the day of transplantation up to day 10, following a previously described protocol (17).

Immunizations

SRBC (10^9 in 800 μl of sterile PBS) were injected i.p. at the day of transplantation. Keyhole limpet hemocyanin (KLH; Sigma, St. Louis, MO) was injected either i.p. (2 mg in 800 μl of sterile PBS) at the indicated time points or in the footpad (50 μg emulsified in 400 μl of CFA) at the day of transplantation.

Detection of circulating CTLA4Ig

CTLA4Ig in sera was detected using a sandwich ELISA. Plates (Nunc Maxisorp, Nalge Nunc International, Naperville, IL) were coated overnight at 4°C with a hamster anti-murine CTLA4-specific mAb (4F10, kindly provided by Dr. J. Bluestone, Chicago, IL) (50 μl at 5.6 μg/ml). Plates were blocked with a solution of PBS, 0.1% Tween, and 1% BSA, and then washed and incubated for 2 h at 37°C with serial dilutions of rat serum in blocking buffer. After washing, either a peroxidase-conjugated goat anti-human IgG (Byosis, Compiegne, France) or goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) was added and incubated for 2 h at 37°C. The reaction was developed using ABTS (Boehringer Mannheim, Mannheim, Germany), and the absorbance of duplicate samples read at 405 nm. CTLA4Ig, either mouse CTLA4 and the constant domains of human IgG1, or mouse CTLA4 and the constant domains of mouse IgG1 diluted in rat serum were used as standards to quantitate serum levels in treated animals. The ELISA detection limit was 1 ng/ml.

Immunohistology

Immunohistology was performed in cryostat sections, as previously described (15). To detect CTLA4Ig in tissues, sections were subsequently incubated (60 min) with a biotin-conjugated rat IgG-absorbed $F(ab')_2$ goat anti-human Fc portion of the IgG Ab (Jackson ImmunoResearch), or hamster mAb anti-murine CTLA4 (4F10). Tissues probed with the mAb were then incubated with a biotin-conjugated rat IgG-absorbed anti-hamster IgG Ab (60 min; Vector Laboratories, Burlingame, CA). Sections were incubated with HRP-conjugated streptavidin (45 min; Vector Laboratories), revealed (5 min) with very intense purple (VIP) substrate (Vector Laboratories), and counterstained by incubation with hematoxylin and lithium chloride.

Immunohistological analysis of infiltrating leukocytes was performed at day 5 after transplantation using mouse mAb: a mixture of two anti-leukocyte CD45 mAbs (OX1 and OX30), anti-monocyte/macrophage CD68 (ED1), anti-$\alpha\beta$ TCR (R.7.3), anti-CD4 (W3/25); anti-CD8 α-chain (OX8), anti-monomorphic class II MHC Ags (OX6), anti-CD25 (OX39) (all from European Cell Culture Collection (ECACC), Wiltshire, U.K.), and an irrelevant mouse mAb (3G8, anti-human CD16). Slides were then incubated with a biotin-conjugated anti-mouse Ig Ab (60 min; Vector Laboratories), followed by HRP-conjugated streptavidin (45 min; Vector Laboratories) and VIP substrate. Quantification was performed by the point-counting technique (18). Briefly, positive cells were counted using a square grid in the eyepiece of the microscope on 15 high power (×400) fields of each slide and expressed as the percentage of the area of biopsy occupied by cells.

Quantitative RT-PCR

Heart samples at day 5 after transplantation were immediately frozen in liquid nitrogen and stored at −80°C until use. Total RNA was isolated using the acid-guanidium phenol-chloroform method, and 10 μg of mRNA was reverse transcribed using the Moloney murine leukemia virus reverse-transcriptase kit (Life Technologies, Paisley, U.K.) (15). Transcript levels for cytokines and hypoxanthine phosphoribosyltransferase (HPRT) were quantified using real-time quantitative PCR and the SYBR green DNA dye (ABI Prism 7700; Perkin-Elmer Applied Biosystems, Foster City, CA) (19). Primer sequences were as follows: IFN-γ, 5′-CAGCTCTGCCTCATG GCC-3′ (sense) and 5′-GATTCTGGTGACAGCTGGTG-3′ (antisense); IL-13, 5′-AGCAACATCACACAAGACCAG-3′ (sense) and 5′-CACAACT GAGGTCCACAGCT-3′ (antisense); iNOS, 5′-GGAGTGTCAGTGGC TTCCAG-3′ (sense) and 5′-TGGCTCTTGAGCTGGAAGAAG-3′

[3] Abbreviations used in this paper: Ad, recombinant adenovirus; ABTS, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); BN, Brown Norway, GIC, graft-infiltrating cells; HPRT, hypoxanthine phosphoribosyltransferase; iNOS, inducible NO synthase; KLH, keyhole limpet hemocyanin; LEW, Lewis.

(antisense); TGF-β1, 5′-CTACTGCTTCAGCTCCACAG-3′ (sense) and 5′-TGCACTTGCAGGAGCGCAC-3′ (antisense); TNF-α, 5′-CCTTACG GAACCCCCTATATT-3′ (sense) and 5′-GACCCGTAGGGCGATTA CAG-3′ (antisense); HPRT, 5′-TGCTGGATTACATTAAAGCGC-3′ (sense) and 5′-CTTGGCTTTTCCACTTCGC-3′ (antisense).

Results were expressed as the intrasample ratio of cytokine to HPRT mRNA copy numbers.

Proliferative responses against alloantigens, mitogens, and KLH

Spleen and mesenteric lymph nodes were pressed through a stainless steel mesh, and mononuclear cells were isolated using density-gradient centrifugation on Ficoll-Hypaque. T cells were purified from total splenocytes by negative selection using a T cell purification kit (R&D Systems, Abingdon, U.K.). Graft-infiltrating cells (GIC) were isolated by incubating finely minced heart allografts in 4 ml of collagenase D (2 mg/ml; Boehringer Mannheim, Indianapolis, IN) (30 min at 37°C), followed by passage through a stainless steel mesh and density-gradient centrifugation on Ficoll-Hypaque. Cells were resuspended in culture medium consisting of RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 1 mM sodium pyruvate, 1% nonessential amino acids, and 5×10^{-5} M 2-ME (all from Sigma). Dendritic cells were enriched from spleen fragments digested with collagenase D (2 mg/ml) for 15 min at 37°C and in the presence of 10 μM EDTA during the last 5 min. The cell suspension was washed twice and resuspended in 5 μM EDTA-PBS containing 2% heat-inactivated FCS at 4°C, at $1–2 \times 10^8$ cells/ml. Four milliliters of this suspension were layered onto 4 ml of 14.5% (w/v) metrizamide (grade I; Sigma) and centrifuged for 13 min at $1500 \times g$ at 4°C. Low density cells were recovered, resuspended at 10^7 cells/ml, and cultured overnight in complete medium containing rat IL-4 and human GM-CSF. Nonadherent cells were gently harvested and contained on average 70% of dendritic cells. Total splenocytes, purified T cells, or GIC were seeded (10^5 cells/well) onto round-bottom 96-well plates (Nunc, Naperville, IL) in triplicate cultures and evaluated for their proliferative response against irradiated dendritic cells (5×10^4 cells/well) or Con A (12.5 μg/ml). Cells were cultured for 3 and 5 days, and for the final 8 h of culture, 1 μCi [^{3}H]thymidine deoxyribose was added to each well and thymidine incorporation was quantified using a scintillation counter.

Proliferation against KLH was analyzed in popliteal lymph node cells from naive or transplanted animals injected with either noncoding or CTLA4Ig-coding adenoviruses. Seven days after injection of KLH (at day 0) in the footpad, lymph node cells were cultured (3×10^5 cells/well, 3 days) with KLH (25 μg/ml and decreasing doses) and pulsed with 1 μCi [^{3}H]thymidine deoxyribose.

Detection of alloantibodies, anti-SRBC, and anti-KLH Abs

LEW.1W or BN splenocytes (2×10^6 cells/ml) were cultured with Con A (Sigma) at 7 μg/ml in complete medium for 72 h. Viable blasts were harvested after a Ficoll-Hypaque density-gradient centrifugation and incubated (30 min at 4°C) with heat-inactivated serum (30 min at 56°C), serially diluted in PBS. Cells were then washed and incubated with either FITC-coupled donkey anti-rat IgG (H+L) (Jackson ImmunoResearch), or FITC-coupled goat anti-rat IgM (Jackson ImmunoResearch). For detection of anti-SRBC Abs, serially diluted sera (heat inactivated) were incubated with SRBC, and developed using a sheep-absorbed FITC-coupled donkey anti-rat IgG or mouse mAbs directed against rat κ-chain (MARK-1), rat IgG1 (MARG1–2), rat IgG2a (MARG2a-7), or rat IgG2b (MARG2b-3) (provided by Dr. D. Lattine, Brussels, Belgium), followed by incubation with a FITC-conjugated rat Ig-absorbed F(ab′)$_2$ goat anti-mouse Ig Ab (Jackson ImmunoResearch). Serum levels of anti-donor, anti-third party, or anti-SRBC Abs were determined by cytofluorometry (FACScalibur; Becton Dickinson, San Jose, CA) and reported as the mean channel fluorescence at a dilution of 1/10 (highest dilution resulting in maximal signal in the sera of immunized untreated controls).

Anti-KLH Abs were detected by ELISA. Plates (Immulon 1; Dynatech Laboratories, Chantilly, VA) were coated overnight at 4°C with 50 μl of KLH (10 μg/ml). The blocking, washing steps, and the incubation of serially diluted sera were performed as mentioned above. A peroxidase-conjugated donkey anti-rat IgG (H+L) (Jackson ImmunoResearch) was added and incubated for 2 h at 37°C. The reaction was developed using ABTS (Boehringer Mannheim).

Anti-adenovirus Abs were analyzed in sera diluted 1/20, 1/100, and 1/1000, as previously described (15).

Statistical analysis

Statistical significance was evaluated using a one-way ANOVA test and Kaplan-Meier analysis for graft survival.

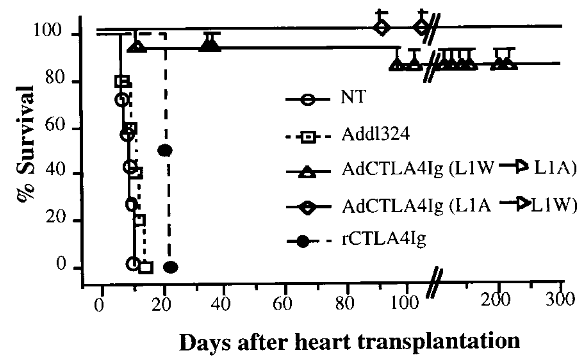

FIGURE 1. Permanent survival of cardiac allografts treated with AdCTLA4Ig. Untreated LEW.1A recipients were transplanted with LEW.1W hearts (day 0) that were either nontransduced (9 ± 1, $n = 7$) or transduced (10^{10} PFU) with the noncoding adenovirus Addl324 (10.8 ± 1.2, $n = 5$) or AdCTLA4Ig (>100 days survival in 90% of the grafts, $n = 20$). A group of LEW.1W animals was transplanted with LEW.1A hearts transduced with AdCTLA4Ig (>100 days survival, $n = 3$). †, Animals sacrificed with well-functioning grafts for analysis of immune responses. Two animals were treated for 10 days with 50 μg/day i.p. of murine rCTLA4Ig from day 0 after transplantation.

Results

Adenovirus-mediated gene transfer of CTLA4Ig indefinitely prolongs cardiac allograft survival

To evaluate the effect of CTLA4Ig produced by the graft on allograft survival, we performed adenovirus-mediated gene transfer into the myocardium using a previously published method (9, 10). We have previously shown that cellular transduction is largely limited to focal areas of cardiac tissue, with low or undetectable transduction of liver, lungs, and spleen (10).

The mean survival time ± SD of cardiac allografts injected with 10^{10} PFU of the noncoding adenovirus Addl234 (10.8 ± 1.2, $n = 5$) was indistinguishable from that of control untreated hearts (9 ± 1, $n = 7$) (Fig. 1). Cardiac allografts injected with 10^{10} PFU of AdCTLA4Ig showed indefinite survival (>100 days in 90% of the recipients) in both the LEW.1W to LEW.1A combination and in the LEW.1A to LEW.1W combination (which otherwise reject between days 7 and 9) (Fig. 1). This indicates that inhibition of graft rejection by gene transfer of CTLA4Ig was not restricted to a single recipient MHC haplotype.

Daily systemic administration of rCTLA4Ig (50 μg) in the LEW.1W to LEW.1A combination during 10 days moderately prolonged allograft survival (up to a maximum of 21 and 22 days) (Fig. 1).

These results show that adenovirus-mediated gene transfer of CTLA4Ig into the heart allowed permanent graft survival, and that this was not due to a particular susceptibility of the LEW.1W to LEW.1A strain combination used in this study since administration of rCTLA4Ig only moderately prolonged graft survival.

Detection of CTLA4Ig

CTLA4Ig expression was analyzed in the sera at different times after gene transfer, using an ELISA (Fig. 2). Levels of CTLA4Ig were higher (between 25 and 150 μg/ml) at days 5 and 30 after gene transfer than at later time points. Nevertheless, most animals showed levels of CTLA4Ig above 30 μg/ml 60 and 90 days after gene transfer. All animals tested between days 120 and 160 after gene transfer showed levels between 5 and 10 μg/ml and of 0.5–4 μg/ml between 200 days and more than 1 year after gene transfer. Animals injected daily with rCTLA4Ig at transplantation for 10

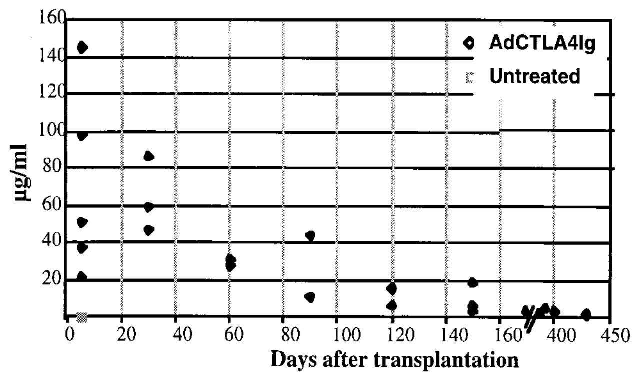

FIGURE 2. Detection of CTLA4Ig in serum after adenovirus-mediated gene transfer. Serum from animals transplanted with untreated grafts (n = 13)or grafts transduced with AdCTLA4Ig were harvested at the indicated time points and analyzed by ELISA for CTLA4Ig detection.

days showed levels of CTLA4Ig in the sera of 1.5 and 3 μg/ml at day 5, 0.1 and 0.4 μg/ml at day 15, and undetectable levels at day 20 after transplantation.

CTLA4Ig was detected by immunohistology in hearts that were injected with AdCTLA4Ig and harvested at days 5 and 120 after gene transfer, but was undetectable in Addl324-injected hearts (Fig. 3, *A–C*). CTLA4Ig immunoreactivity was widespread throughout the whole graft. Higher levels of CTLA4Ig were detected in grafts harvested at early time points, but hearts still expressed CTLA4Ig at least 120 days after gene transfer. Five days after transplantation, CTLA4Ig was also strongly detected by immunohistology in the red pulp and B cell areas of the spleen from animals transplanted with AdCTLA4Ig-transduced hearts, but not in spleens from controls (Fig. 3, *D* and *E*). CTLA4Ig was also detected in mesenteric lymph nodes from animals transplanted with AdCTLA4Ig-transduced hearts (Fig. 3*F*).

Long lasting CTLA4Ig expression could be due to the inhibition of anti-adenovirus immune responses by CTLA4Ig itself. Anti-adenovirus Ab levels were analyzed by ELISA in the serum of untreated animals or of recipients bearing grafts treated with either AdCTLA4Ig or noncoding adenoviruses (10^{10} PFU). None of the animals (n = 5) in the group treated with AdCTLA4Ig showed detectable levels of anti-adenovirus Abs (at 1/20 serum dilution: mean ± SD of 0.115 ± 0.017 OD; ranging from 0.098 to 0.149) at any dilution tested. These values were identical to those observed in the sera of animals not injected with adenoviruses (n = 2, 0.130 ± 0.010, ranging from 0.120 to 0.141). In contrast, three of four animals that received Addl324 showed detectable anti-adenovirus Ab levels (0.318 ± 0.102, ranging from 0.145 to 0.408).

These results indicate that CTLA4Ig was still being produced long after gene transfer, and that this was associated to an inhibition of humoral anti-adenovirus immune responses. The presence of CTLA4Ig was not restricted to the graft because it was also detected in the serum, spleen, and lymph nodes.

Immunohistological analysis of leukocytes infiltrating the grafts

Total leukocytes, mononuclear cell subsets, and activation markers were quantitatively analyzed in cardiac grafts 5 days after transplantation and gene transfer (Fig. 4). Hearts injected with AdCTLA4Ig or controls showed comparable infiltration by total leukocytes ($OX1^+$ and $OX30^+$), monocytes/macrophages ($ED1^+$), $\alpha\beta$T ($R73^+$), $CD4^+$ ($W3/25^+$), and $CD8^+$ ($OX8^+$) cells. In spite of this, hearts treated with AdCTLA4Ig showed a significant reduction in the number of cells expressing molecules involved in allorejection, such as MHC class II molecules ($OX6^+$) or the α-chain of the IL-2R ($OX39^+$) (Fig. 4) compared with untreated hearts or those injected with the noncoding adenovirus.

These results suggests that local expression of CTLA4Ig does not affect the total numbers and subset composition of graft-infiltrating leukocytes, but can modulate the expression of activation markers associated with graft rejection.

Analysis of cytokine expression in the grafts

Quantification of mRNA levels for cytokines and iNOS expressed within transplanted hearts 5 days after transplantation showed that hearts treated with AdCTLA4Ig contained significantly reduced

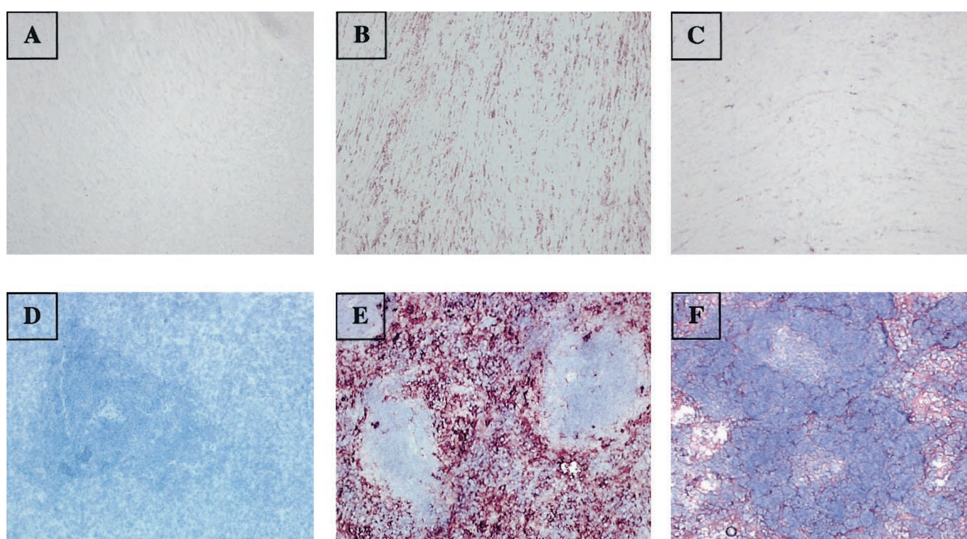

FIGURE 3. Presence of CTLA4Ig in tissues after adenovirus-mediated gene transfer. Tissue cryostat sections were analyzed by immunohistology using anti-human IgG Abs. LEW.1W heart grafts transduced with noncoding Addl324 adenoviruses and harvested 5 days later (*A*) showed lack of detectable CTLA4Ig. LEW.1W heart grafts transduced with AdCTLA4Ig showed the presence of more abundant labeling at day 5 (*B*) than at day 120 (*C*) after gene transfer. Spleens from animals transplanted with hearts transduced with noncoding Addl324 adenoviruses (*D*) or AdCTLA4Ig (*E*) were analyzed 7 days after transplantation. CTLA4Ig was detected in the red pulp and B cell areas of spleens. Lymph nodes from animals transplanted with hearts transduced with AdCTLA4Ig (*F*) harvested 7 days after transplantation also showed the presence of CTLA4Ig.

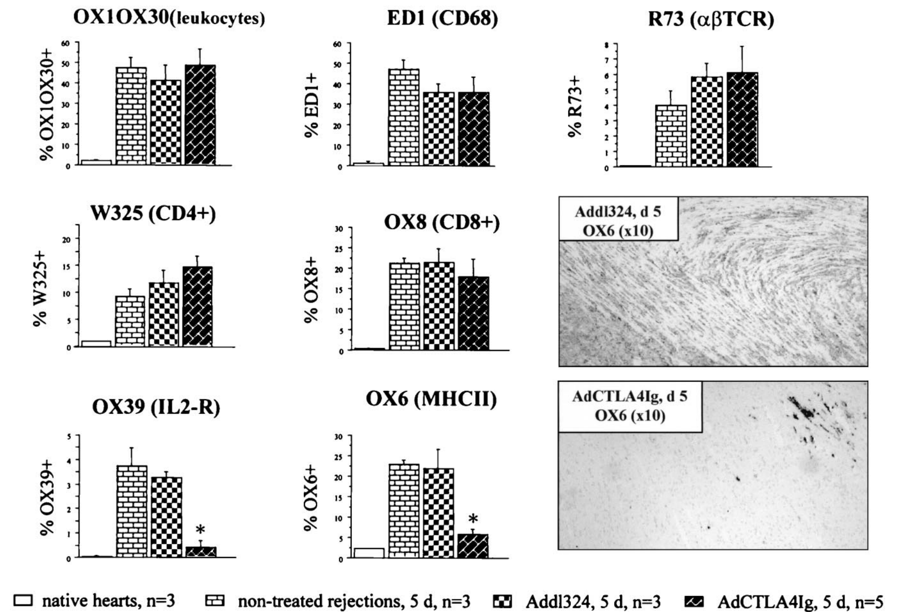

FIGURE 4. Quantitative immunohistochemical analysis of heart leukocyte infiltration at day 5 after transplantation and gene transfer. Native hearts or cardiac grafts either untreated or injected with 10^{10} PFU of Addl324 or AdCTLA4Ig-Ig were harvested and frozen, and cryostat sections were incubated with mAbs. Tissues were analyzed morphometrically, and data are expressed as the percentage area of biopsy occupied by cells ± SE. Photomicrographs correspond to cryostat sections of Addl324- or AdCTLA4Ig-treated grafts analyzed 5 days after transplantation with OX6 anti-MHC class II mAb. *, $p < 0.05$ as compared with untreated or Addl324-treated animals.

transcript levels for IFN-γ, iNOS, and TGF-β1, whereas IL-13 levels were increased in three of six grafts, but this increase was not statistically significant (Fig. 5).

These results suggest that CTLA4Ig expression induced a local modification in the production of cytokines with an inhibition of type 1 (IFN-γ) cytokine production and in some animals an increased type 2 (IL-13) production. The reduction in iNOS gene expression furthermore suggests a decreased macrophage and/or endothelial cell activation.

Inhibition of the MLR responses of graft-infiltrating cells and splenocytes, but not of lymph node cells from animals bearing AdCTLA4Ig-treated grafts

To analyze the effect of adenovirus-mediated CTLA4Ig expression on cellular allogeneic responses, analysis of MLR responses with

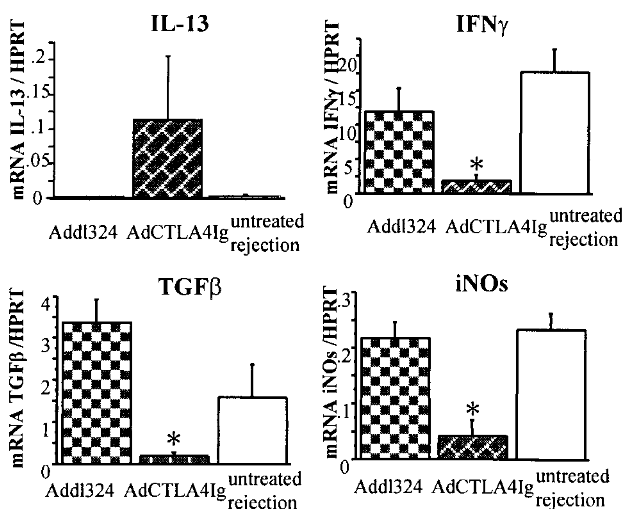

FIGURE 5. Analysis of cytokine mRNA accumulation within grafts by quantitative RT-PCR. Total RNA was extracted from cardiac grafts 5 days after gene transfer, retrotranscribed, and analyzed by quantitative real-time PCR. Results were expressed as the intrasample ratio of cytokine to HPRT mRNA copy numbers. Data represent the mean ± SE from four to six rats per group and from experiments repeated at least twice. *, $p < 0.05$ as compared with untreated or Addl324-treated animals.

cells harvested from grafts (Fig. 6*A*), spleens (Fig. 6*B*), or lymph nodes (Fig. 6*C*) was performed 5 days after transplantation. Proliferative responses were evaluated against donor LEW.1W, third party BN dendritic cells, or Con A.

In comparison with GIC from grafts either untreated or treated with the noncoding adenovirus, GIC from AdCTLA4Ig-treated grafts showed a profound inhibition of proliferation not only in response to donor cells, but also to third party cells or Con A (data not shown) after 3 (Fig. 6*A*) or 5 days of culture (data not shown). Incubation with IL-2 increased proliferative responses of both groups of animals, but did not reverse the inhibition observed in MLR from animals bearing AdCTLA4Ig-treated grafts (Fig. 6*A*).

Compared with controls, splenocytes from animals bearing AdCTLA4Ig-treated grafts also showed an inhibition of proliferation in response to donor or third party cells and Con A after 3 (Fig. 6*B*) or 5 days of culture (data not shown). Addition of IL-2 only slightly increased their proliferation (Fig. 6*B*). Interestingly, T cells purified from splenocytes of animals bearing AdCTLA4Ig-treated grafts showed comparable proliferative responses to those of T cells from control animals (Fig. 6*B*). The lack of proliferation in response to Con A in the absence of IL-2 is explained by the fact that purified T cells are unable to proliferate in response to Con A in the absence of APC or exogenous IL-2. Addition of IL-2 did not increase the proliferation of T cells against alloantigens in either group, but induced their proliferation to Con A (Fig. 6*B*).

In contrast to splenocytes, mesenteric lymph node cells from animals bearing AdCTLA4Ig-treated grafts showed proliferative responses to alloantigens and to Con A comparable with those of animals either untreated or treated with noncoding adenoviruses, in the presence or absence of IL-2 after 3 days (Fig. 6*C*) or 5 days of culture (data not shown).

Because GIC and recipient splenocytes from animals bearing AdCTLA4Ig-treated grafts showed staining for CTLA4Ig (Fig. 3), most likely reflecting binding to B7 molecules on APC, we evaluated the capacity of both cell populations to act as APCs and stimulate an MLR response. Splenocytes from LEW.1W animals depleted of dendritic cells proliferated in the presence of splenocytes from either untreated or noncoding adenovirus-treated LEW.1A animals, but showed >90% inhibition of proliferation in response to splenocytes from animals bearing AdCTLA4Ig-treated grafts (data not shown). As APCs from AdCTLA4Ig-treated

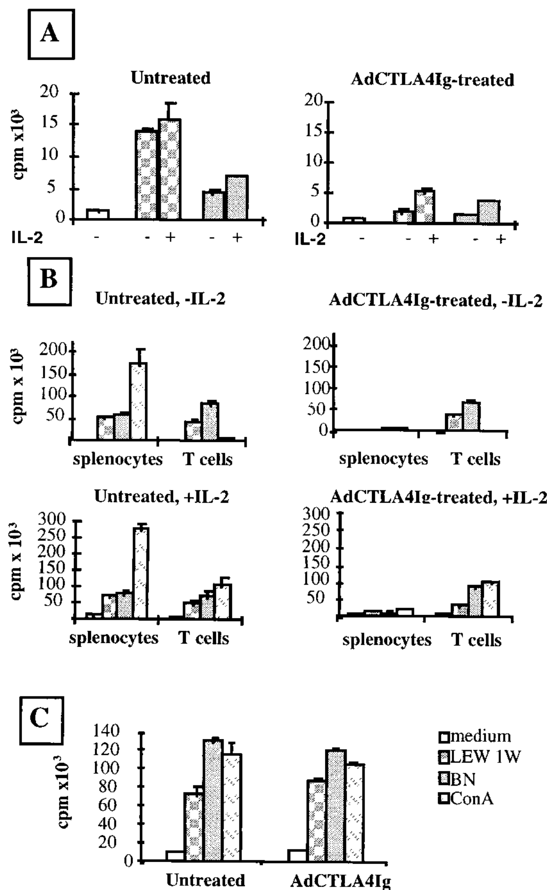

FIGURE 6. Inhibition of one-way MLR responses in recipients grafted with AdCTLA4Ig-transduced hearts. MLR responses of cells harvested from graft (*A*), spleens (*B*) (T cells were purified from splenocytes), or lymph nodes (*C*) from the same LEW.1A recipient grafted with either untreated or AdCTLA4Ig-treated hearts were analyzed 5 days after transplantation. Cellular allogeneic responses against first party LEW.1W or third party BN dendritic cells, in the presence or absence of IL-2, were analyzed after 3 days of culture. Results are expressed as the mean ± SD cpm of one representative animal from two tested for GIC, five tested for splenocytes, and three for lymph node cells.

LEW.1A animals were not capable of stimulating LEW.1W T cells, the inhibition of MLR responses observed using LEW.1W APCs as stimulators and LEW.1A splenocytes as responders can be either explained by the absence of costimulation (blockade of B7) or by a suppressive activity of APCs.

We hypothesized that inhibition of proliferation could be due to the presence of CTLA4Ig in MLR supernatants that could be either produced or released by recipient APC, and that would also block costimulation by donor APCs. CTLA4Ig levels were low in MLR supernatants from GIC (2.2 and 2.4 ng/ml) and undetectable in MLR supernatants from splenocytes. Since the minimal concentration needed to inhibit >90% of MLR responses is 1 μg/ml (17), the absence or very low concentrations of CTLA4Ig present in the MLR supernatant from AdCTLA4Ig-treated recipients cannot explain the inhibition of proliferative responses due to direct Ag presentation.

Altogether, these results show that despite the presence of CTLA4Ig in spleen and lymph nodes (see Fig. 3), allogeneic and mitogenic proliferative responses were inhibited in some (graft and spleen), but not all (lymph nodes) lymphoid compartments. Because direct recognition by T cells of allodeterminants on donor APCs was at least in part present (i.e., T cells responded to donor Ags), the inhibition of MLR responses against donor Ags is not explained by T cell anergy and suggests that at least a part of the alloreactive clones have not been deleted. The concomitant inhibition of donor, third party, and mitogen-driven proliferative responses favors the existence of suppressive interactions between T cells and non-T cells in the graft and in the spleen, resulting in nonspecific suppression.

Inhibition of alloantibody production in AdCTLA4Ig-treated recipients

Anti-allogeneic humoral responses of AdCTLA4Ig-treated recipients were evaluated by cytofluorometric analysis at different time points (Fig. 7). When compared with untreated rejected hearts, recipients treated with AdCTLA4Ig showed virtually undetectable levels of IgM and IgG Abs against LEW.1W at every time point analyzed up to 90 days after transplantation. These findings were confirmed by immunohistological analysis of grafts more than 100 days after transplantation, which showed the absence of detectable alloantibody deposition (data not shown).

Expression of CTLA4Ig by transduced hearts results in inhibition of immune responses against cognate Ags

We then determined whether the immunosuppressive effect detected within animals treated with AdCTLA4Ig was specific for anti-donor humoral immune responses or whether it also affected unrelated cognate Ags. We thus analyzed immune responses against SRBC injected immediately after transplantation or against KLH injected at 30, 60, or 120 days after allotransplantation and gene transfer. All animals that received Addl324 or AdCTLA4Ig were successively immunized against SRBC at day 0 and against KLH at day 60. Anti-SRBC levels in animals transplanted with AdCTLA4Ig-transduced grafts were comparable with those of nonimmunized controls (for IgM and all IgG subclasses) and lower than those of recipients treated with noncoding adenovirus (Fig.

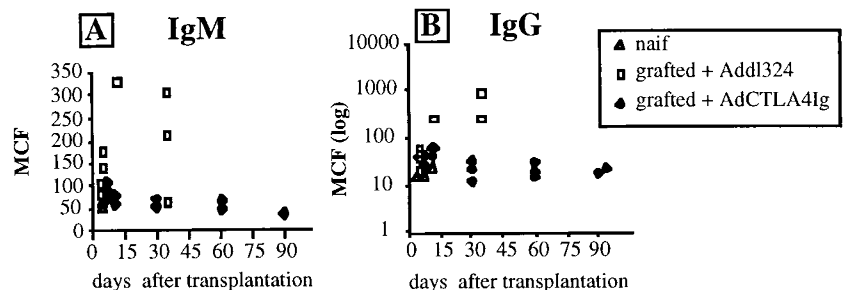

FIGURE 7. Suppression of alloantibody production in recipients grafted with AdCTLA4Ig-transduced hearts. Sera from LEW.1A recipients grafted with LEW.1W hearts were collected at the indicated time points. Serial dilutions were incubated with LEW.1W Con A blasts and analyzed by cytofluorometry for the presence of IgM (*A*) or IgG (*B*) alloantibodies. Results are expressed as mean channel fluorescence (MCF) at the dilution of 1/10.

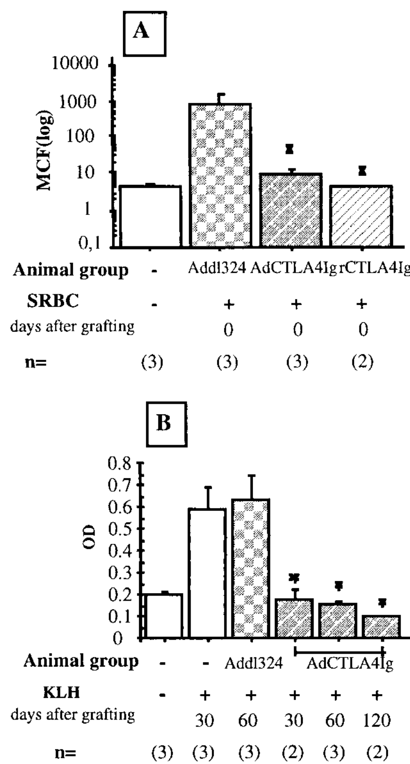

FIGURE 8. Suppression of Ab production against cognate Ags in recipients grafted with AdCTLA4Ig-transduced hearts. Rats grafted with hearts transduced with Addl324 or AdCTLA4Ig, or injected with rCTLA4Ig were immunized with SRBC at the day of transplantation. Levels of anti-SRBC Abs (IgG2a) in sera were analyzed by cytofluorometry at day 15 after transplantation (*A*). At later time points after transplantation (days 30, 60, and 120), KLH (2 mg/kg) was injected, and levels of anti-KLH Abs in sera were analyzed by ELISA 10 days later (*B*). *, $p < 0.05$ as compared with untreated or Addl324-treated immunized animals.

8*A*). Recipients injected with rCTLA4Ig (from day 0 to 10) also showed complete suppression of anti-SRBC Ab production.

Animals grafted with AdCTLA4Ig-treated hearts and injected in the footpad with KLH at transplantation showed 40–50% less cells in draining lymph nodes, and secondary in vitro proliferative responses against KLH were inhibited between 60 and 75% as compared with control animals grafted with Addl324-treated hearts (data not shown).

To analyze the humoral immune response in animals transplanted with AdCTLA4Ig-transduced grafts at later time points, KLH was injected and levels of anti-KLH Abs were analyzed 10 days later (Fig. 8*B*). Animals treated with AdCTLA4Ig showed inhibition in the production of anti-KLH Abs compared with controls. Recipients injected with rCTLA4Ig and injected with KLH at day 30 after transplantation showed high levels of anti-KLH Abs, comparable with those of untreated immunized controls (data not shown).

These results indicate that recipients of hearts transduced with AdCTLA4Ig showed a systemic suppression of immune responses that lasted longer than that observed in recipients treated with rCTLA4Ig.

Graft survival and systemic humoral immune responses at doses of AdCTLA4Ig lower than 10^{10} PFU

Since the injection of 10^{10} PFU of AdCTLA4Ig allowed indefinite graft survival in all recipients, but induced systemic immunosuppression of humoral immune responses, we performed gene transfer with lower doses of AdCTLA4Ig and analyzed graft survival and Ab production to cognate Ags (Table I). Recipients transplanted with grafts transduced with doses of AdCTLA4Ig lower than 10^{10} PFU showed the presence of CTLA4Ig in serum that were lower than those found in animals that had received 10^{10} PFU of AdCTLA4Ig (see Fig. 2), but the levels obtained did not strictly correlate with the amount of AdCTLA4Ig injected into the grafts. Animals transplanted with grafts injected with 5×10^9 PFU showed indefinite survival and complete inhibition of anti-SRBC (immunization at day 0) and anti-KLH Ab production (immunization at day 90). One of two grafts injected with 2.5×10^9 or 1.25×10^9 PFU showed prolonged survival, but were ultimately rejected (at days 17 and 43, respectively), whereas the remaining graft in each group was permanently accepted. Recipients who received the two lowest doses showed complete inhibition of anti-SRBC Ab production, but showed a partial response against KLH.

In conclusion, decreasing the doses of AdCTLA4Ig below 10^{10} PFU enabled prolongation of heart survival, but reduced the efficiency in achieving indefinite graft acceptance. Systemic humoral immune responses were suppressed at early time points, but partially present at later time points.

Donor-specific tolerance in recipients with long surviving grafts after adenovirus-mediated CTLA4Ig gene transfer

To evaluate whether recipients with long-term surviving grafts showed donor-specific tolerance, we grafted these animals with skin or a second heart from LEW.1A (syngeneic), LEW.1W (first party donor), or Fischer (third party donor) origin animals (Table II).

Skin from LEW.1A syngeneic animals was permanently accepted, whereas skin from LEW.1W first party donors was rejected with the same kinetics as for skin from unrelated third party donors (Table II).

The skin and the heart show different rejection mechanisms (20). In some models of tolerance induction toward vascularized organs, a dichotomy between rejection of first party-matched second skin graft and acceptance of a second vascularized graft has

Table I. *Dose-response effect of AdCTLA4Ig*[a]

AdCTLA4Ig (pfu)	*n*	CTLA4Ig in Serum (μg/ml)	Survival (days)	Anti-SRBC Inhibition (%)	Anti-KLH Inhibition (%)
1.25×10^9	2	3, 1.54	17, >100	>95	52, 76
2.5×10^9	2	1.7, 1.2	43, >100	>95	52, 78
5×10^9	2	1.7, 4.3	>100	>95	83, 91

[a] AdCTLA4Ig was delivered into cardiac grafts (LEW.1W to LEW.1A combination) at the indicated doses. CTLA4Ig was quantified in serum at day 10 after gene transfer using a sandwich ELISA. SRBC and KLH were administered at days 0 and 90, respectively, after gene transfer. Anti-SRBC Abs and anti-KLH Abs were quantified by cytofluorimetry and ELISA, respectively. Levels for both kinds of Abs were compared to those (100% reactivity) of positive control animals, i.e. those immunized with the Ag and treated with noncoding Add1324 adenoviruses.

Table II. *Donor- and organ-specific tolerance in recipients with AdCTLA4Ig-treated long surviving hearts*[a]

Donor Strain (MHC haplotype)	Second Graft Survival (days)	
	Skin	Heart (neck)
LEW 1A (RT1[a])	>50	ND
LEW 1W (RT1[u])	8, 9, 10	>150, >150, >150
Fischer (RT1[lv1])	8, 9, 9	18, 20, 20

[a] LEW.1A recipients bearing AdCTLA4Ig-transduced LEW.1W long-term (>150 days) surviving hearts (grafted in the abdomen) received a second graft, either skin or a cardiac graft (grafted onto the neck). Donor strains were LEW.1A (syngeneic control), LEW.1W (same haplotype as the first cardiac graft), or Fischer (third-party control).

been described (3). Therefore, we performed second cardiac grafts in recipients with long surviving grafts (>150 days). In contrast to skin, second hearts from LEW.1W donors were indefinitely accepted (>150 days, $n = 3$) (Table II). Hearts from third party Fischer donors were rejected (19 ± 1 day, $n = 3$), despite prolonged survival as compared with survival in untreated LEW.1A recipients (8 ± 1, $n = 3$). All first LEW.1W grafts were functional >150 days after grafting of first or third party second hearts. Rejection of LEW.1W skin induced rejection of the first LEW.1W heart graft in one of three recipients 45 days after skin transplantation.

To further analyze the mechanisms underlying permanent graft acceptance in recipients with long surviving AdCTLA4Ig-transduced hearts, we performed an analysis of MLR responses from splenocytes and purified T cells against either first or third party donor dendritic cells or Con A. When compared with controls (200 days after untreated rejections), splenocytes from animals bearing AdCTLA4Ig-treated permanently accepted grafts showed 50–55% inhibition of MLR responses against donor-matched dendritic cells after 3 (Fig. 9) or 5 days of culture (data not shown). Proliferation against third party cells was reduced by 10–15%, and proliferation against Con A was not inhibited. Addition of IL-2 to the MLR cultures with dendritic cells of donor origin significantly increased the MLR response of splenocytes from recipients with permanently accepted grafts, whereas proliferation of splenocytes from control animals was unchanged. Addition of IL-2 to the MLR cultures with dendritic cells of third party origin did not modify the proliferative responses of either group. Proliferation induced by Con A was increased in all animals in the presence of IL-2. Importantly, and as observed at day 5, T cells purified from splenocytes from recipients with long surviving AdCTLA4Ig-treated grafts showed MLR responses identical to those of T cells from untreated controls (data not shown).

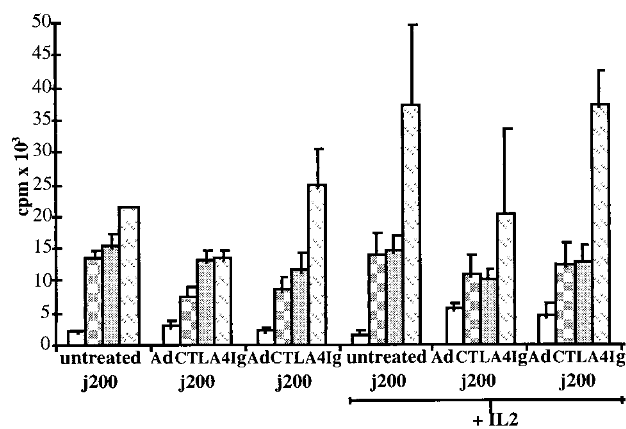

FIGURE 9. Donor-specific hyporesponsiveness of one-way MLR responses in recipients with AdCTLA4Ig-treated permanently accepted hearts. Recipients were grafted with untreated hearts (rejected at day 10) or hearts transduced with AdCTLA4Ig (permanently accepted, two animals), and proliferative responses were analyzed 200 days after transplantation. Cellular responses against first party LEW.1W, third party BN dendritic cells, or Con A, in the presence or absence of IL-2, were analyzed after 3 days of culture. Results are expressed as the mean ± SD cpm. □, Medium; ▨, LEW.1W; ▩, BN; ▧, Con A.

These results show that recipients bearing AdCTLA4Ig-transduced grafts show donor-specific tolerance toward a vascularized organ, but not to skin. Permanent heart acceptance was dependent on active immune mechanisms, as demonstrated by in vivo and in vitro experiments.

Discussion

Blocking T cell costimulatory signals has been successfully applied to inhibit immune responses in autoimmune diseases and transplantation (1). In particular, blockade of CD28-B7 by a single administration of rCTLA4Ig prolongs survival of vascularized grafts and can lead to permanent acceptance in models such as kidney and islet allotransplantation (1). However, this is not the case in rat heart allotransplantation models (2, 5, 6, 17). Even prolonged (7 or up to 21 days) administration of low (0.05 mg) or high doses (0.5 mg) of rCTLA4Ig did not extend heart allograft survival (from 7 days in controls to 30 days) compared with that obtained after a single administration (2, 17). Indefinite heart survival with rCTLA4Ig has only been achieved by simultaneous administration of donor cells (2, 5), anti-CD4 (3), or anti-CD40L mAb (4, 21). Adenovirus-mediated gene transfer of CTLA4Ig in the liver resulted in permanent acceptance of liver allografts (11), whereas systemic gene transfer in a heart allotransplantation model moderately prolonged allograft survival (12). The present study shows that intragraft expression of CTLA4Ig following adenovirus-mediated gene delivery results in indefinite heart survival, whereas administration of rCTLA4Ig for 10 days prolonged heart survival for up to 22 days. As compared with administration of rCTLA4Ig, the indefinite heart survival obtained with AdCTLA4Ig is probably the consequence of higher serum levels of CTLA4Ig that persist for longer. Alternatively, it is possible that production of CTLA4Ig within the graft allows better bioavailability, and thus a more effective blockade of B7 molecules expressed by graft-infiltrating macrophages, dendritic cells, and activated endothelial cells (22).

CTLA4Ig was detected throughout in AdCTLA4Ig-transduced hearts, despite the fact that the gene transfer by intramyocardial injection only allows cell transduction to areas that are relatively restricted to injection points, as detected when using a transgene product retained within cells such as nlslacZ (10). It is likely that cells positively stained with anti-CTLA4Ig Abs were transduced cardiomyocytes producing the protein and B7-positive cells (i.e., infiltrating leukocytes and endothelial cells) coated with CTLA4Ig. The presence of CTLA4Ig in serum for long periods after gene transfer can only be explained by continuous production of CTLA4Ig by transduced cells. Inhibition of anti-adenovirus immune responses and long-term transgene expression have already been described using adenoviruses coding for CTLA4Ig (23). Since we observed inhibition of anti-adenovirus Ab production in recipients of AdCTLA4Ig-treated grafts, it is very likely that prolonged expression of CTLA4Ig after gene transfer in the graft is due to blunted anti-adenovirus immune responses. CTLA4Ig serum levels in rats, after transplantation and gene transfer, were

close to those that have been shown to prolong liver allograft survival after gene transfer with adenoviruses (11). CTLA4Ig was also detected in areas of lymphoid tissues rich in B7-positive cells, and therefore probably represents binding of CTLA4Ig to recipient B7-expressing cells. It is also likely that among these cells, APCs of donor origin that normally migrate from the heart to the spleen (24) have also interacted with CTLA4Ig, either in the graft or during the transit from the graft to the spleen.

Gene transfer with AdCTLA4Ig did not eliminate the prominent mononuclear cell infiltrate observed in untreated allogeneic hearts at day 5 after transplantation, a finding that we and others have described in various tolerance-inducing models with or without the use of CTLA4Ig (2, 6, 10, 11, 22, 25, 26). As already described in previous studies (6, 11, 27) in long surviving grafts using CTLA4Ig, permanently accepted hearts in our study also showed the presence of infiltrating leukocytes, albeit at lower levels than at day 5 after transplantation. As in previous studies (6, 11, 27), these hearts did not show signs of chronic rejection vascular disease (data not shown). The presence of a leukocyte infiltrate indicates the persistence of cellular responses against the grafted tissue, despite the absence of rejection, and most likely reflects the establishment of tolerogenic mechanisms. The crucial issue is the anti-inflammatory and immunodeviating activity of these leukocytes as opposed to the proinflammatory and tissue-destructive potential of leukocytes present in grafts that will be rejected. This is shown at the phenotypic level by the lower expression of CD25 and MHC class II Ags, and functionally by the different pattern of cytokine expression and by the suppressed proliferative responses of GIC from AdCTLA4Ig-treated grafts compared with controls.

In several studies, administration of CTLA4Ig has been associated with a switch in the production from type 1 to type 2 cytokines within the grafts (6, 11, 28) as well as with an inhibition in the production of iNOS and TGF-β1 (28). In our study, gene transfer of CTLA4Ig induced decreased IFN-γ mRNA levels within the grafts, which was also confirmed at the protein level (data not shown), and a nonsignificant increase in IL-13 transcripts, reflecting a local decrease in the production of type 1 cytokines and possibly of the proinflammatory potential of graft-infiltrating leukocytes. Furthermore, the reduction in iNOS mRNA levels also suggests that local activation of macrophages and endothelial cells (the two major sources of iNOS) may also be reduced. Despite the fact that gene transfer of TGF-β1 in the heart induces indefinite graft acceptance (9) and that tolerance induced by donor-specific blood transfusion is dependent on TGF-β1 production (9), expression of TGF-β1 mRNA was reduced in grafts treated with AdCTLA4Ig. These results provide evidence for the existence of diverse tolerance mechanisms and suggest that various experimental models may activate different mechanisms.

Systemic administration of rCTLA4Ig has been described to efficiently inhibit in vivo priming against nominal Ags (29–32), but this phenomenon has never been analyzed after gene transfer of CTLA4Ig in the transplantation setting (11, 12). CTLA4Ig produced after adenovirus-mediated gene transfer into the graft resulted in immunosuppressive effects on Ags other than donor alloantigens, as shown by the inhibition of alloantibody production and lymph node proliferation against nominal Ags (SRBC or KLH). Gene transfer with lower doses of AdCTLA4Ig resulted in complete inhibition of humoral immune responses at early time points (SRBC), partial inhibition of humoral immune responses at late time points (KLH), and a lower frequency of permanently accepted grafts. These results indicate that to obtain permanent graft acceptance, high levels and/or long-term expression of CTLA4Ig are necessary. Immunosuppression during long-term CTLA4Ig protein administration or after adenovirus-mediated expression could represent a risk for the recipient. This risk could be limited through the use of an adenovirus coding for CTLA4Ig under the transcriptional control of an inducible promoter. This would allow exploration of the minimal time required to establish the tolerogenic mechanisms responsible for indefinite heart allograft survival.

Although CTLA4Ig induces anergy in vitro (defined as absence of T cell proliferation), CTLA4Ig in vivo results in prolonged unresponsiveness through the action of inhibitory mechanisms and not through anergy induction (27, 31, 32). In vivo treatment with CTLA4Ig in transplantation models has either shown no inhibition (21, 30) or moderate (<50%) inhibition of subsequent MLR responses (2, 17, 33) or against mitogens (30). Our results clearly indicated that GIC and splenocyte proliferative responses at early time points (5 days) were not only profoundly inhibited against donor-matched alloantigens, but also to third party cells and mitogens. T cells derived from splenocytes proliferated normally, indicating that T cells were not anergic, but rather partially deleted and/or functionally inhibited. Inhibition of MLR responses indicates that alloantigen presentation by both recipient APCs and donor APCs is inhibited. Responses to Con A, which are heavily dependent on APC signaling, were also inhibited in these organs. Blockage of T cell signaling by recipient APCs could be due to the binding of CTLA4Ig, but this is unlikely to be the mechanism responsible for the blockade of stimulator APCs in the MLR because levels of CTLA4Ig from MLR supernatants were undetectable or too low to mediate this effect and anti-CD28 mAb did not reverse the inhibition of proliferative responses (data not shown). Furthermore, despite that APCs from lymph nodes showed binding of CTLA4Ig as for spleen, MLR or Con A proliferative responses were not inhibited, strongly suggesting that the inhibition of proliferation observed for GICs and spleen cells was not only due to B7 blockade by CTLA4Ig. It is unlikely that this is explained by differences in tissue distribution of CTLA4Ig because treatment with CTLA4Ig has been shown to modulate lymph node immune responses (30), and in recipients with AdCTLA4Ig-treated grafts we demonstrated binding of CTLA4Ig in lymph nodes and inhibition of secondary proliferative responses of lymph node cells against KLH. This dichotomy of proliferative responses against alloantigens and mitogens between GIC and splenocytes vs lymph node cells may be explained by the presence of donor APCs in the graft and in the spleen, but not in lymph nodes after heart transplantation (24). Alternatively, alloreactive T cells may not be found in the lymph nodes because activated T cells lose expression of CD62L (explaining why they cannot home in lymph nodes) and gain expression of VLA4 (explaining their homing to the graft through interaction with VCAM-1 expressed by activated endothelial cells) (34). The APC/T interaction in the context of costimulation blockade would generate a suppressive environment (35) in the graft or spleen, but not in lymph nodes, by production of suppressive factors by either the APCs, the T cells, or both, as it has been recently described in other models (35, 36). We have observed identical inhibition of MLR responses with splenocytes and GICs, but not lymph nodes in tolerant animals after donor-specific blood transfusion (unpublished results).

In contrast to the profound and nonspecific inhibition of proliferative responses at early time points, splenocyte proliferation in recipients with long surviving grafts showed donor-specific hyporesponsiveness, suggesting a recovery of immune responses or the existence of weaker but donor-specific regulatory mechanisms active during the maintenance phase of tolerance in our model. Our data confirm previously published results in which administration of CTLA4Ig resulted in tolerance of pancreatic islets through the activity of suppressive mechanisms (27). Previous reports have

also shown that in vivo tolerance to a cognate Ag is not due to anergy, but rather to a dual mechanism of decreased expansion of Ag-reactive cells and decreased functional activity of remaining cells (32).

Importantly, our in vitro results suggesting the existence of donor-specific tolerance mechanisms in long surviving recipients are supported by our in vivo results showing acceptance of first party matched second hearts and rejection of third party hearts. Furthermore, the in vitro results showing that anergy or complete T cell clonal deletion was not the mechanism of heart tolerance were supported in vivo by the rejection of skin of donor origin. In previous allograft tolerance models induced by administration of rCTLA4Ig, the recipients showed donor-specific tolerance when rechallenged with the same organ as the first graft, but rejected second skin grafts (2, 6, 11, 27, 37). One of three long surviving recipients rejected their heart graft after rejection of skin from first party donors. As in our study, rejection of skin from first party origin induced rejection of the first heart graft in one of four animals in the only previous study that addressed this issue (2). Acceptance of second hearts and rejection of skin, as well as triggering of rejection of long surviving recipients, can be explained by the presence of skin-specific Ags, by the higher content of APCs in skin, and by strong direct alloantigen presentation (20).

Adenovirus-mediated CTLA4Ig gene transfer in our heart transplantation model allowed definition of some of the drawbacks and advantages of this type of therapeutic strategy in transplantation. Circulating CTLA4Ig resulted in systemic immunosuppression of T cell-dependent humoral immune and proliferative (lymph node cells) responses against cognate Ags as well as inhibition of proliferation (MLR and Con A) of splenocytes. Certain immune responses were nevertheless conserved, such as proliferation of lymph node cells against alloantigens or Con A.

Additionally, adenovirus-mediated CTLA4Ig resulted not only in very efficient induction of donor-specific tolerance, but also revealed potentially new peripheral mechanisms of tolerance induction such as suppressive APC/T interactions in the graft or spleen, but not in lymph nodes. Both gene transfer methods and vectors need to be improved to obtain spatial and temporal regulation of gene expression, and therefore determine the minimum length of time during which CTLA4Ig is needed for tolerance induction and to reduce nonspecific immunosuppression, while maintaining a safe and efficient gene therapy strategy.

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