

# Prolonged Blockade of CD40-CD40 Ligand Interactions by Gene Transfer of CD40Ig Results in Long-Term Heart Allograft Survival and Donor-Specific Hyporesponsiveness, But Does Not Prevent Chronic Rejection<sup>1</sup>

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Previous work on blockade of CD40-CD40 ligand interaction in mice and primates with anti-CD40 ligand mAbs has resulted in a moderate prolongation of allograft survival without the development of true allograft tolerance. In this study, we show in rats that adenovirus-mediated gene transfer of CD40Ig sequences into the graft resulted in prolonged (>200 days) expression of CD40Ig and in long-term (>300 days) survival. Recipients expressing CD40Ig displayed strongly (>90%) inhibited mixed leukocyte reactions and alloantibody production at early (days 5 and 17) and late time points (>100 day) after transplantation, but showed limited inhibition of leukocyte infiltration and cytokine production as evaluated by immunohistology at early time points (day 5). Recipients of long-surviving hearts showed donor-specific hyporesponsiveness since acceptance of second cardiac allografts was donor specific. Nevertheless, long-term allografts (>100 days) displayed signs of chronic rejection vasculopathy. Occluded vessels showed leukocyte infiltration, mainly composed of CD4<sup>+</sup> and CD8<sup>+</sup> cells, macrophages, and mast cells. These recipients also showed antidonor CTL activity. Recipients expressing CD40Ig did not show nonspecific immunosuppression, as they were able to mount anticognate immune responses that were partially inhibited at early time points and were normal thereafter. We conclude that gene transfer-mediated expression of CD40Ig resulted in a highly efficient inhibition of acute heart allograft rejection in rats. This treatment induced donor-specific inhibition of certain alloreactive mechanisms in the short-, but not the long-term, which resulted in long-term survival of allografts concomitant with the development of chronic rejection. *The Journal of Immunology*, 2002, 168: 1600–1609.

**T**ransplantation has achieved impressive rates of graft survival, with up to 90% of transplanted kidneys surviving after the first year (1). These results have been obtained through the use of immunosuppressive drugs, which unfortunately also result in increased rates of cancer, opportunistic infections, as well as side effects on organ function, and for certain among these drugs, blockade of tolerance induction. Furthermore, these drugs do not prevent chronic rejection, resulting in 50% of kidneys being functional 10 years after transplantation (2). The use of new bioreagents, such as Abs, cytokines, and soluble receptors, to block specific steps of the immune response may prevent acute rejection,

decrease side effects, and eventually lead to tolerance toward the graft, thus eliminating chronic rejection. Some of the drawbacks of these bioreagents come from the fact that systemic administration, the most widely used route of administration, results in systemic and nonspecific immunosuppression as well as in poor bioavailability due to the short  $t_{1/2}$  of many of these types of molecules.

Gene transfer of immunoregulatory molecules to the graft is an alternative to their systemic delivery. Production of these molecules by the graft may allow for more localized rather than systemic effects (although this needs to be specifically evaluated when using secreted molecules), and may also result in an increased bioavailability as a consequence of their continuous production (3). Prolongation of allograft survival has been previously described using gene transfer of immunoregulatory molecules (4–9).

Priming, expansion, and differentiation of T cells play a central role in graft rejection. Priming of naive T cells depends on a first set of signals delivered by the TCR after interaction with peptides presented within MHC molecules on the surface of APCs. Optimal priming and activation are obtained by a series of interactions between accessory molecules expressed by both APCs and T cells that reciprocally activate both cell types. Among these, the CD40-CD40 ligand (CD40L)<sup>5</sup> signaling pathway has been shown to be

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<sup>5</sup> Abbreviations used in this paper: CD40L, CD40 ligand; AdCD40Ig, adenovirus coding for CD40Ig; BN, Brown Norway; HO-1, heme oxygenase-1; iNOS, inducible NO synthase; IP, infectious adenoviral particle; KLH, keyhole limpet hemocyanin; LEW, Lewis; MLR, mixed leukocyte reaction.

an essential component in the initiation and maintenance of thymus-dependent humoral and cellular immune responses (10). CD40L is a member of the TNF family, found on activated CD4<sup>+</sup> cells and a fraction of CD8<sup>+</sup> T cells as well as on mast cells, eosinophils, platelets, and macrophages (10, 11). Its counterreceptor, CD40, is a member of the TNFR superfamily and is expressed on dendritic cells, macrophages, B cells, and endothelial cells, among several other cell types (10, 12). CD40-CD40L interaction results in the reciprocal activation of APCs and B cells via CD40 and the activation of T cells via CD40L (10).

Blockade of CD40-CD40L interactions by the use of anti-CD40L mAbs in mice (12–18) and primates (19, 20) has resulted in prolongation of allograft survival. However, in only some of these studies, a fraction of the recipients showed long-term engraftment (12, 17, 19, 20). In recipients with long-surviving grafts, evidence for true donor-specific tolerance (as defined by permanent graft survival with acceptance of second donor, but not third party-derived grafts in the absence of immunosuppression and of chronic rejection (21, 22)) has not been formally proven. Furthermore, the absence of CD40-CD40L interactions did not prevent the development of chronic rejection in mice, but the mechanisms implicated have not been clearly established (18, 23–25).

The aim of this study was to obtain prolonged blockade of CD40-CD40L interactions through gene transfer-mediated expression of CD40Ig in a rat model of heart allograft rejection and to evaluate its effects on acute and chronic rejection mechanisms.

Our results show that high and prolonged CD40Ig expression after gene transfer resulted in long-term acceptance of heart allografts. Inhibition of mixed leukocyte reaction (MLR) and alloantibody responses and conservation of anticognate Ag immune responses were also observed. Despite donor-specific acceptance of second grafts, long-term surviving allografts displayed chronic rejection lesions and antidonor CTLs, indicating that certain allogeneic immune responses were not inhibited by blockade of CD40-CD40L interactions.

## Materials and Methods

### *Animals, heart transplantation, and administration of anti-CD40L Abs*

Two hundred- to 250-gram inbred male Lewis 1W (LEW.1W, haplotype RT1<sup>b</sup>) or Brown Norway (BN, haplotype RT1<sup>b</sup>) rats were used as donors, and Lewis 1A (LEW.1A, haplotype RT1<sup>b</sup>) rats were used as recipients (CERJ, Le Genest St. Isle, France) (9). All sentinel rats housed in the same colony were specific pathogen free. Heterotopic cardiac allografts were placed into the abdomen (first grafts) or into the neck (second grafts). Graft survival was monitored daily by palpation. Rejection (mean survival time) was defined as total cessation of cardiac beating and was confirmed by direct examination.

### *Recombinant adenovirus coding for CD40Ig and gene transfer into the heart*

Previous results have shown that expression of the cDNA sequences coding for the extracellular portion of mouse CD40 fused to the coding sequences of the constant domains of human IgG1 interacts with CD40L (26, 27). The CD40Ig cDNA (kindly provided by P. Lane, Birmingham University, Birmingham, U.K.) was placed under the transcriptional control of a murine CMV promoter (28). An adenovirus coding for CD40Ig (AdCD40Ig) was generated, propagated, and purified according to standard protocols, as previously described (28–30). The noncoding adenoviral vector Add324 has been previously described (30). Recombinant adenoviruses were titered using a replication center assay. The protocol, originally described for the titration of adenovirus-associated vectors (31), was modified to allow the quantification of infectious adenoviral particles (IP). Briefly, 293 cells were seeded at  $8 \times 10^4$  cells/well in 48-well plates. The next day, they were infected with serially diluted vectors. Cells were trypsinized 36 h later and filtered through a Zetaprobe membrane (Bio-Rad,

Hercules, CA). Filters were then soaked in 0.5 M of NaOH, 1.5 M of NaCl for 5 min, neutralized in 1 M of Tris-HCl (pH 7.2)  $\times$  SSC, and finally incubated with a fluorescein-labeled nucleic probe hybridizing to the DNA-binding protein gene. Quantification of IP was performed by counting the number of spots (corresponding to individual viral replication events) on infected 293 cells. Importantly, quantification by replication center assay yielded titers equivalent to infectious units (determined by immunofluorescence using an anti-DNA-binding protein Ab). 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  $10^9$  IP). For gene transfer into the heart, recombinant adenoviruses ( $5 \times 10^{10}$  IP in 250  $\mu$ l) were slowly injected into the apex and ventricular walls of the heart at four different points, as previously described in detail (9, 32).

### *Detection of CD40Ig*

Serum CD40Ig was detected using a sandwich ELISA. Plates (Nunc Maxisorb; Nalge Nunc International, Roskilde, Denmark) were coated overnight at 4°C with rabbit anti-human IgG CH2 domain Ab (Dako, Glostrup, Denmark) (50  $\mu$ l at 10  $\mu$ g/ml). Plates were incubated (1 h at 37°C) with blocking buffer (PBS containing 0.1% Tween and 10% rat serum) and then incubated with serial dilutions of rat serum in PBS containing 0.1% Tween (2 h at 37°C). After washing, a biotin-conjugated rat IgG-adsorbed donkey anti-human IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) was added and incubated for 1 h at 37°C. Plates were then incubated with HRP-conjugated streptavidin (45 min at 37°C; Vector Laboratories, Burlingame, CA), the reaction was developed using 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (Boehringer-Mannheim, Mannheim, Germany), and the absorbance of duplicate samples read at 405 nm. Purified CTLA4Ig (containing the same human IgG Fc fragment as CD40Ig) (26) diluted in rat serum was used as a standard to quantitate serum levels of CD40Ig in treated animals. The ELISA detection limit was 0.02 ng/ml.

CD40Ig in tissues was detected by incubating (60 min) cryostat sections with a biotin-conjugated rat IgG-adsorbed F(ab')<sub>2</sub> donkey anti-human Fc portion Ab (Jackson ImmunoResearch Laboratories). Sections were then incubated with HRP-conjugated streptavidin (45 min; Vector Laboratories), revealed (5 min) with very intense purple substrate (Vector Laboratories), and counterstained with hematoxylin and lithium chloride. CD40Ig binding to leukocytes was analyzed by FACS using anti-human IgG Abs.

### *Histological analysis*

Immunohistology was performed on cryostat sections, as previously described in detail (9, 30), with the exception that for certain markers (CD8 $\beta$ , CD161) blockade of endogenous peroxidases with hydrogen peroxide was omitted. Immunohistological analysis of infiltrating leukocytes was performed at day 5 after transplantation using the following mouse mAbs: 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 $\alpha$  chain (OX8), anti-monomorphic class II MHC Ags (OX6), anti-CD25 (OX39), anti-CD8 $\beta$  chain (3.4.1), anti-CD161 (NKR-P1, 3.2.3), and IL-4 (OX81) (all from European Cell Culture Collection, Wiltshire, U.K.); and anti-CD86 (B7.2; BD Pharmingen, Franklin Lakes, NJ). IFN- $\gamma$  (R&D Systems, Abingdon, U.K.) and CD40L (clone AH.F5; kindly provided by C. Benjamin, Biogen, Boston, MA) were analyzed using hamster mAbs. Rabbit Abs were used to detect inducible NO synthase (iNOS; Transduction Laboratories, Lexington, KY) and heme oxygenase-1 (HO-1; Stressgen Biotechnologies, San Diego, CA) expression. An irrelevant mouse mAb (3G8, anti-human CD16), hamster, or rabbit sera were used as negative controls. Slides were then incubated with biotin-conjugated anti-mouse, anti-hamster, and anti-rabbit Abs from Jackson ImmunoResearch Laboratories. Binding of these Abs was detected by incubation with HRP-conjugated streptavidin and very intense purple substrate (Vector Laboratories). Tissue sections were counterstained with hematoxylin and lithium carbonate.

Histological assessment of long-surviving hearts was performed on paraffin-embedded sections stained with H&E-saffron. Vascular lesions (percentage obstruction, leukocyte infiltration, media lesions) were analyzed in at least 10 medium-size vessels. The presence of mast cells and eosinophils was evaluated after May-Grünwald-Giemsa staining of cryostat sections, and the number of positive cells per field ( $\times 20$  objective) was counted.

### *Immunizations*

SRBC ( $10^9$  in 800  $\mu$ l of sterile PBS) were injected i.v. on the day of transplantation. Keyhole limpet hemocyanin (KLH; Sigma-Aldrich, St.

Louis, MO) was injected at >100 days after transplantation in the footpad (50  $\mu$ g emulsified in 200  $\mu$ l of CFA).

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

Alloantibodies were analyzed by cytofluorometry (FACSCaliber; BD Biosciences, San Jose, CA) after transplantation by incubating serially diluted heat-inactivated serum with splenocytes cultured with Con A for 3 days, followed by incubation with human IgG-adsorbed biotin-conjugated F(ab')<sub>2</sub> goat anti-rat IgG ( $\gamma$ -chain specific) or rat IgM ( $\mu$ -chain specific) (Jackson ImmunoResearch Laboratories) and with FITC-coupled streptavidin. Results were reported as mean channel fluorescence for each serum dilution. Serum levels of anti-SRBC Abs were determined at day 17 after immunization by incubation of serially diluted heat-inactivated serum with SRBC, and Ab binding was detected as described above.

Anti-adenovirus Abs were detected by ELISA using a previously described technique (30). Briefly, plates (Nunc Maxisorb) were coated overnight at 4°C with adenoviruses (10<sup>9</sup> particles in 50  $\mu$ l of PBS), fixed with formaldehyde (1%, 20 min), blocked, washed, and incubated with serial dilutions of sera (all with PBS containing 0.1% Tween and 0.1% BSA). Human IgG-adsorbed biotin-conjugated donkey anti-rat IgG ( $\gamma$ -chain specific) or rat IgM ( $\mu$ -chain specific) (Jackson ImmunoResearch Laboratories) was added and incubated for 2 h at 37°C. Binding was detected as described above for detection of CD40Ig by ELISA. Anti-KLH Abs were detected at day 9 after immunization by ELISA. Plates (Nunc Maxisorb) were coated overnight at 4°C with KLH (50  $\mu$ l at 10  $\mu$ g/ml). The blocking and washing steps, the incubation of serially diluted sera, and detection of IgG or IgM binding were performed as described above.

#### Proliferative responses against alloantigens, Con A, and KLH

Spleen and mesenteric lymph nodes were pressed through a stainless steel mesh into RPMI 1640. The cells were pelleted and resuspended in Tris-ammonium chloride buffer (0.83% NH<sub>4</sub>Cl, 5 mM Tris buffer, pH 7.2) at room temperature for 10 min to lyse erythrocytes. T cells were purified from total splenocytes by negative selection using a T cell purification kit (R&D Systems, Abingdon, U.K.). Cells were resuspended in culture medium consisting of RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM of L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 1 mM of sodium pyruvate, 1% nonessential amino acids, 1% HEPES, and 5  $\times$  10<sup>-5</sup> M of 2-ME (all from Sigma-Aldrich). Dendritic cells were enriched from LEW.1W or third-party BN spleen fragments digested with collagenase D (2 mg/ml) for 20 min at 37°C and in the presence of 10 mM of EDTA for the last 5 min. The cell suspension was washed twice and resuspended in 0.5 mM of EDTA-PBS containing 2% heat-inactivated FCS at 4°C. A total of 4 ml of this suspension was layered onto 4 ml 14.5% (w/v) Nycodenz AG (Life Technologies, Paris, France) and centrifuged for 15 min at 2800  $\times$  g at 4°C. Low density cells were recovered, resuspended at 5  $\times$  10<sup>6</sup> cells/ml, and cultured overnight in complete medium. Nonadherent cells were gently harvested and used as dendritic-enriched cells. Responder cells were seeded (10<sup>5</sup> cells/well) onto round-bottom 96-well plates (Nunc) in triplicate cultures and evaluated for their proliferative response against irradiated dendritic cells (5  $\times$  10<sup>4</sup> cells/well) or Con A (12.5  $\mu$ g/ml). Cells were cultured for 3 and 5 days, and for the final 8 h of culture 1  $\mu$ Ci of [<sup>3</sup>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 Add1324 or CD40Ig-coding adenoviruses. Nine days after injection of KLH in the footpad, lymph node cells were cultured (3  $\times$  10<sup>5</sup> cells/well) in flat-bottom 96-well plates (Nunc) for 3 days with KLH (25  $\mu$ g/ml and decreasing doses) and pulsed with 1  $\mu$ Ci of [<sup>3</sup>H]thymidine deoxyribose for the last 8 h of culture.

#### CTL assay

Splenocytes were isolated and used directly as effector cells. <sup>51</sup>Cr (100  $\mu$ Ci for 1 h at 37°C)-labeled LEW.1W, BN, and LEW.1A spleen Con A (2  $\mu$ g/ml, in the presence of IL-2 at 100 U/ml for 3 days) blasts were used as target cells. The effector and target cells were plated in triplicate round-bottom 96-well microtiter plates (10<sup>4</sup> target cells/well) at E:T ratios ranging from 100:1 to 12.5:1 in the culture medium defined above. After incubation (6 h at 37°C), the plates were centrifuged, <sup>51</sup>Cr was measured using a beta counter, and the percentage of specific lysis was calculated as 100  $\times$  (cpm experimental release - spontaneous release)/cpm (maximal release - spontaneous release). Maximal release, cells incubated with 1%

SDS; spontaneous release, cells incubated with medium. Spontaneous release was always <10%.

#### Statistical analysis

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

## Results

### Expression of CD40Ig results in long-term heart allograft acceptance

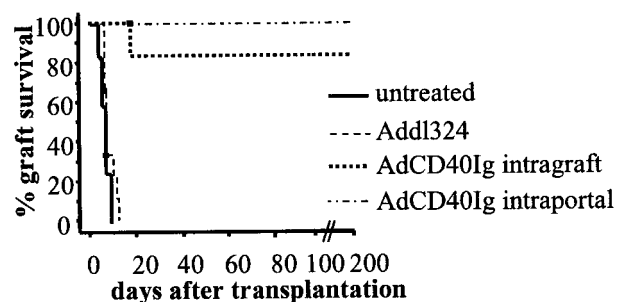
Rejection (mean survival time  $\pm$  SD) of LEW.1W control untreated hearts (9  $\pm$  1,  $n$  = 8) by LEW.1A recipients was indistinguishable from hearts treated with the noncoding adenovirus Add1324 (10.8  $\pm$  1.2,  $n$  = 9) (Fig. 1). A single administration of AdCD40Ig within the graft resulted in long-term (>200 days,  $n$  = 17) graft survival in 83% of the recipients, and in the remaining cases graft rejection occurred only after day 20 (Fig. 1). Administration of CD40Ig into the portal vein, which resulted in a large majority of adenoviruses being trapped and expressed within the liver and lungs, also resulted in long-term allograft survival (>200 days,  $n$  = 3).

These results show that adenovirus-mediated CD40Ig gene transfer directly into the transplanted heart or at a distant site resulted in efficient abrogation of acute heart rejection.

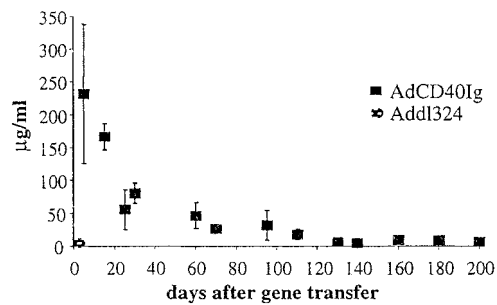
### CD40Ig expression following gene transfer

We analyzed the presence of CD40Ig in serum using a specific ELISA. After gene transfer into the graft, CD40Ig levels in the serum were between 147 and 229  $\mu$ g/ml at day 5 (Fig. 2). Levels declined thereafter, but remained high, between 6.8 and 13.5  $\mu$ g/ml, 160 days after gene transfer. Add1324 or untreated animals did not show detectable levels of CD40Ig (<0.02 ng/ml) (Fig. 2). At day 17 after gene transfer, transplanted animals that received AdCD40Ig into the portal vein displayed levels of CD40Ig in serum comparable with those observed in recipients in which AdCD40Ig was directly injected into the graft (51–100  $\mu$ g/ml,  $n$  = 3).

Analysis of CD40Ig binding in tissues was performed by immunohistology using an anti-human IgG Ab 5 days after gene transfer. Intense and widespread staining was observed in AdCD40Ig-treated grafts (Fig. 3A). Lymph nodes from recipients of AdCD40Ig-treated grafts showed strong staining of cells in the marginal sinus, paracortical area, and medullary cords



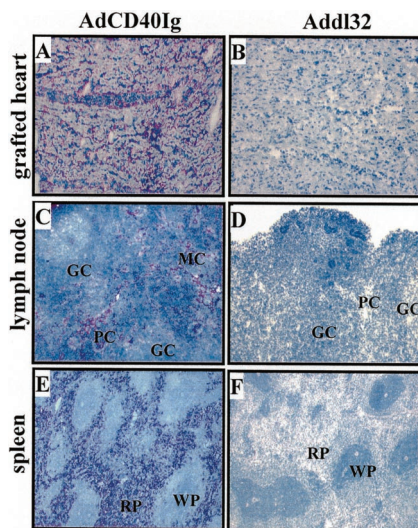
**FIGURE 1.** Permanent survival of cardiac allografts treated with AdCD40Ig. LEW.1A recipients were transplanted with LEW.1W hearts that were either untreated ( $n$  = 8) or transduced ( $5 \times 10^{10}$  IP) with the noncoding adenovirus Add1324 ( $n$  = 9) or AdCD40Ig (>100 days survival in 83% of the grafts,  $n$  = 17). A group of LEW.1A animals was transplanted with LEW.1W hearts, and AdCD40Ig ( $5 \times 10^{10}$  IP) was administered via the portal vein ( $n$  = 3).



**FIGURE 2.** Detection of CD40Ig in serum after adenovirus-mediated gene transfer. Serum from animals transplanted with grafts transduced ( $5 \times 10^{10}$  IP) with noncoding Addl324 adenoviruses (hatched symbol) or AdCD40Ig (filled symbols) was harvested at the indicated time points and analyzed by ELISA for CD40Ig detection ( $n = 6-13$  per time point). CD40Ig levels in Addl324 or nontreated animals were below the ELISA detection limit (0.02 ng/ml).

(Fig. 3C). Splens from recipients with AdCD40Ig-treated grafts displayed intense and homogeneous labeling in the red pulp, and dispersed cells in T and B cell areas (Fig. 3E). The graft, lymph nodes, and spleen of Addl324-treated recipients did not show staining with the anti-human IgG Ab (Fig. 3, B, D, and F), confirming the specificity of the signal obtained in tissues from AdCD40Ig-treated recipients.

Expression of CD40L and binding of CD40Ig on CD3<sup>+</sup> and CD3<sup>-</sup> cells present in graft-infiltrating cells and splenocytes were assessed by dual-color FACS analysis. CD40L and CD40Ig in



**FIGURE 3.** Detection of CD40Ig in tissues after adenovirus-mediated gene transfer. Tissue cryostat sections were analyzed by immunohistology using anti-human IgG Abs 5 days after grafting. A, C, and E were harvested from AdCD40Ig-treated recipients, and B, D, and F from Addl324-treated recipients ( $5 \times 10^{10}$  IP). A, LEW.1W heart grafts transduced with AdCD40Ig showed abundant labeling. B, LEW.1W heart grafts transduced with noncoding Addl324 adenoviruses showed no staining. C, Mesenteric lymph nodes from recipients with AdCD40Ig-treated grafts displayed positive cells in paracortical (PC) and medullary cords (MC), but no or few positive cells in germinal centers (GC). D, Lymph nodes from Addl324-treated controls showed no staining. E, Splens from animals transplanted with hearts transduced with AdCD40Ig showed intense labeling in the red pulp (RP) and in isolated cells from the white pulp (WP). F, Splens from Addl324-treated controls displayed no labeling. (A–D objective  $\times 20$ ; E and F objective  $\times 10$ ). Results are from one representative animal of five tested in each group.

graft-infiltrating cells were detected in similar proportions on CD3<sup>+</sup> and CD3<sup>-</sup> cells (19 vs 21%, respectively). In splenocytes, CD40L and CD40Ig were detected on a higher percentage of CD3<sup>-</sup> cells than CD3<sup>+</sup> (12 vs 6%, respectively) (data not shown).

These results indicate that gene transfer with AdCD40Ig leads to the expression of high levels of circulating CD40Ig, resulting in binding to cells not only within the graft, but also in the spleen and lymph nodes.

#### *Decreased graft leukocyte infiltration in AdCD40Ig-transduced grafts*

Analysis of grafted hearts at day 5 after transplantation revealed a moderate decrease in total CD45<sup>+</sup> infiltrating leukocytes, CD68<sup>+</sup> macrophages,  $\alpha\beta$ TCR<sup>+</sup> T, CD4<sup>+</sup>, and MHC class II Ag<sup>+</sup> cells in AdCD40Ig-treated hearts compared with Addl324 (Fig. 4) or untreated grafts (data not shown). CD8<sup>+</sup>, CD25<sup>+</sup>, CD80, CD86, CD28, iNOS, HO-1, and IFN- $\gamma$  positive cells were reduced in some animals, but overall levels were comparable in AdCD40Ig- and Addl324-transduced grafts (data not shown).

#### *Alloantibody responses are inhibited in recipients of AdCD40Ig-treated grafts at early and late time points after transplantation*

Alloantibody levels were analyzed by cytofluorometry in recipients of CD40Ig-treated grafts. Serum IgG alloantibody levels 17 days after transplantation were markedly reduced in recipients of AdCD40Ig-transduced grafts compared with controls treated with Addl324 and were comparable with those of naive animals (Fig. 5A). IgM alloantibodies in AdCD40Ig recipients were significantly decreased compared with those receiving Addl324 at day 17 after transplantation (data not shown).

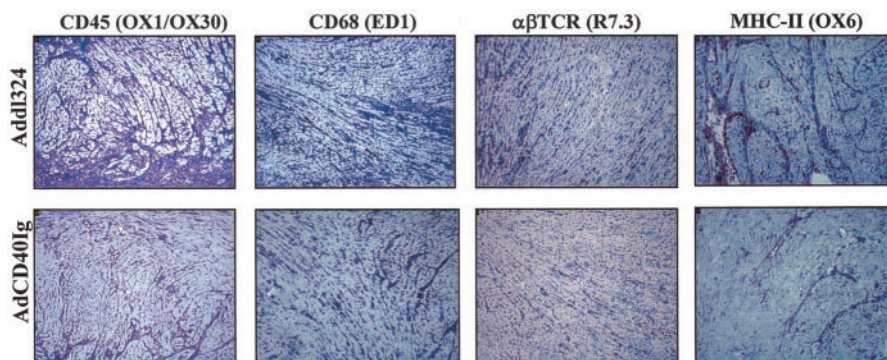
IgG (Fig. 5B) and IgM (data not shown) alloantibody levels in the sera of recipients of CD40Ig-expressing long-surviving grafts were also markedly reduced compared with those receiving Addl324-treated grafts and were comparable with those of naive animals. Deposition of IgM and IgG on long-surviving grafts was comparable with that found on syngeneic grafts (data not shown), as evaluated by immunohistology using serially diluted anti-rat IgM or IgG Abs at saturating and nonsaturating concentrations to enable detection of quantitative differences.

These results indicate that alloantibody responses were efficiently inhibited by CD40Ig expression at early and late time points after transplantation.

#### *Inhibition of splenocyte proliferation in recipients of AdCD40Ig-treated grafts at early and late time points after transplantation*

At 5 or >100 days after transplantation, proliferative responses of splenocytes, T cells purified from splenocytes, and lymph node cells from AdCD40Ig- or Addl324-treated recipients were analyzed after 3 and 5 days of culture, and results were reported for the peak of proliferation. At day 5 after transplantation, splenocytes from recipients of AdCD40Ig-treated hearts displayed, in all cases, very low residual proliferative responses (mean percentage  $\pm$  SD) as compared with Addl324-treated controls (100% responses,  $n = 5$ ) at day 5 (Fig. 6A) and day 3 (data not shown) of culture against LEW.1W donor ( $9 \pm 18$ ,  $n = 9$ ,  $p < 0.001$ ) and third party BN alloantigens ( $6 \pm 12$ ,  $n = 5$ ,  $p < 0.001$ ). Addition of IL-2 only partially reversed the inhibition of splenocyte proliferation against LEW.1W ( $15 \pm 35.5$ ,  $n = 8$ ,  $p < 0.001$  vs Addl324 + IL-2) or BN ( $31 \pm 41$ ,  $n = 8$ ,  $p < 0.001$  vs Addl324 + IL-2) (Fig. 6A), indicating that this inhibition was not due to T cell anergy. Analysis of MLR supernatants did not reveal detectable levels CD40Ig ( $< 0.02$  ng/ml), indicating that in vitro production of CD40Ig did not explain

**FIGURE 4.** Immunohistological analysis of graft-infiltrating leukocytes at early time points after transplantation. AdCD40Ig- or Addl324-injected grafts ( $5 \times 10^{10}$  IP) were harvested at day 5 after transplantation and analyzed by immunohistology with the indicated mAbs. Results are representative of five animals analyzed in each group. Objective  $\times 10$ .



the MLR inhibition. T cells isolated from splenocytes showed partial, but significant decreased proliferative responses against LEW.1W cells vs Addl324 controls ( $70 \pm 7$ ,  $n = 5$ ,  $p < 0.001$ ), but not against BN cells ( $92.4 \pm 12.5$ ,  $n = 5$ ,  $p > 0.05$ ) (Fig. 6B). Proliferation of lymph node cells harvested at day 5 after transplantation from AdCD40Ig-treated recipients was comparable with that of Addl324 controls in three of five animals and only partially inhibited in the other two against donor ( $62 \pm 5.6$ ,  $p < 0.001$ ) and third party ( $8.5 \pm 7.8$ ,  $p > 0.05$ ) alloantigens, and this differential inhibition did not correlate with the levels of CD40Ig in serum (data not shown).

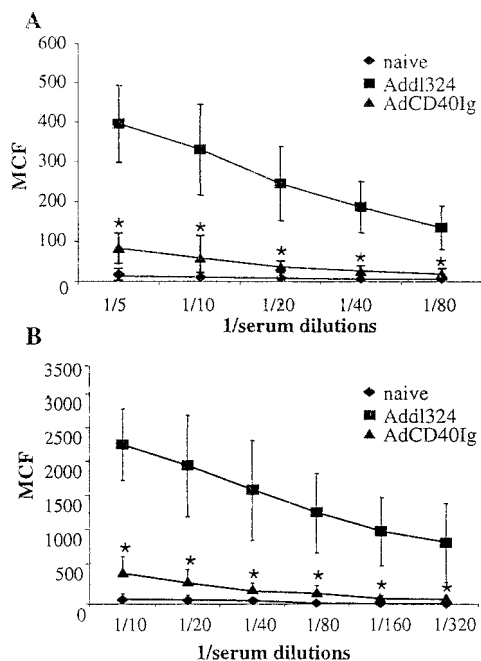
To further explore the mechanisms underlying long-term graft acceptance, we analyzed the proliferative responses of lymphocytes from recipients with long-surviving hearts against

alloantigens. Splenocytes harvested  $>100$  days after transplantation from recipients of CD40Ig-expressing grafts showed very weak proliferation against donor LEW.1W alloantigens in all animals tested ( $9 \pm 12$ ,  $n = 7$ ,  $p < 0.001$ ), and higher against BN third party alloantigens ( $52 \pm 34$ ,  $n = 7$ ,  $p < 0.01$ ) compared with controls at day 5 (Fig. 6C) and at day 3 of culture (data not shown). Purified T cells showed partial inhibition of proliferation against donor LEW.1W cells ( $79 \pm 15$ ,  $n = 7$ ,  $p < 0.01$ ) and against BN third party cells ( $70 \pm 7$ ,  $n = 7$ ,  $p < 0.001$ ) (Fig. 6D). Lymph node cells also showed partial inhibition of MLRs against donor LEW.1W cells ( $62 \pm 24.5$ ,  $n = 7$ ,  $p < 0.001$ ) and against BN third party cells ( $62 \pm 16.5$ ,  $n = 7$ ,  $p < 0.001$ ) (data not shown).

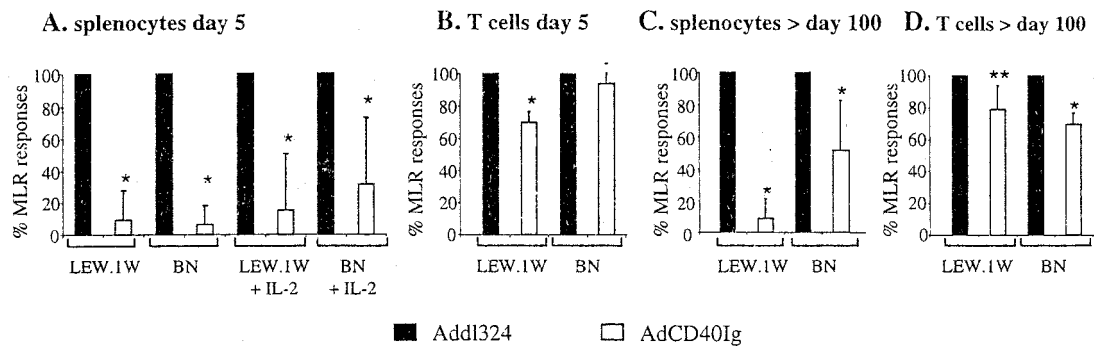
Altogether, these results indicate that inhibition of T cell proliferation was partially dependent on T cell intrinsic allospecific inhibitory mechanisms, but mainly due to the presence of APCs. Donor-specific inhibition of alloantigen-driven proliferation was observed when alloantigens were presented indirectly by recipient APCs (MLRs with splenocytes). Lymphocyte proliferation was less inhibited when alloantigens were either only presented via the direct pathway of allorecognition by donor APCs (MLRs with T cells) or when recipient APCs were present in low numbers (MLRs with lymph node cells).

#### *Donor-specific second graft acceptance in recipients of AdCD40Ig-treated long-surviving grafts showed*

To evaluate whether recipients of long-surviving grafts ( $>100$  days) showed donor-specific mechanisms of graft acceptance, second heart grafts of donor or third party origin were performed. Second hearts of first party LEW.1W (RT1<sup>U</sup>) origin were accepted ( $>150$  days,  $n = 2$ ), whereas third party BN (RT1<sup>P</sup>) grafts were rejected with a slight delay in two recipients (11 and 12 days vs  $9.8 \pm 0.5$ ,  $n = 3$ , in naive controls). These results indicate the existence of donor-specific mechanisms of graft acceptance in recipients of long-surviving grafts after CD40Ig expression. To confirm that graft acceptance was not dependent on high levels of circulating CD40Ig, we harvested the first graft (which was the main site of production of CD40Ig) in one of the two long-surviving recipients 104 days after transplantation of the second graft, and we evaluated CD40Ig levels and second graft survival. CD40Ig levels decreased from  $8 \mu\text{g/ml}$  at the removal of the first graft to  $0.6 \mu\text{g/ml}$  110 days later (214 days after transplantation of the second graft). These concentrations of CD40Ig were not sufficient to inhibit acute rejection (data not shown), indicating that the permanent survival of second grafts of first party donor origin was not dependent on high levels of circulating CD40Ig.



**FIGURE 5.** Decrease IgG alloantibodies at early and late time points after transplantation in recipients of AdCD40Ig-transduced hearts. Serial dilutions of sera from LEW.1A animals nongrafted (naive) or grafted with LEW.1W hearts transduced with the noncoding adenovirus Addl324 or AdCD40Ig were incubated with LEW.1W Con A blasts and analyzed by cytofluorometry for the binding of IgG alloantibodies. A, Sera were collected at day 17 after transplantation (naive,  $n = 3$ ; Addl324,  $n = 4$ ; AdCD40Ig,  $n = 6$ ). B, Sera were collected at day  $>100$  after transplantation (naive,  $n = 3$ ; Addl324,  $n = 4$ ; AdCD40Ig,  $n = 9$ ). Results are expressed as mean channel fluorescence (MCF  $\pm$  SD). \*,  $p < 0.05$  as compared with animals with Addl324-treated grafts, and  $p > 0.05$  as compared with naive animals.



**FIGURE 6.** Inhibition of one-way MLR responses of splenocytes and T cells at early and late time points after transplantation in recipients of AdCD40Ig-transduced hearts. MLR responses of splenocytes or T cells from recipients of either Addl324 (■)- or AdCD40Ig (□)-treated hearts harvested at day 5 or >100 after transplantation were assessed against irradiated first party LEW.1W or third party BN APCs. Results are expressed as the percentage (mean  $\pm$  SD) of cell proliferation of AdCD40Ig-treated rats vs those of Addl324-treated rats considered as 100% (proliferation of cells from Addl324-treated rats ranged between 10,000 and 70,000 cpm). *A*, Proliferation of splenocytes harvested at day 5 after transplantation against irradiated first party LEW.1W or third party BN APCs was analyzed after 5 days of culture in the presence or absence of IL-2. Five to nine animals in each group. *B*, Proliferation of T cells purified from splenocytes harvested at day 5 after transplantation against irradiated LEW.1W or BN APCs was analyzed at the peak of proliferation (day 3 or 5). Five animals in each group. *C*, Proliferation of splenocytes harvested at >100 days after transplantation against irradiated LEW.1W or BN APCs was analyzed after five days of culture. Five to six animals in each group. *D*, Proliferation of T cells purified from splenocytes harvested >100 days after transplantation against irradiated LEW.1W or BN APCs was analyzed at the peak of proliferation (day 3 or 5). Five animals in each group. \*,  $p < 0.001$ , and \*\*,  $p < 0.01$  as compared with animals with Addl324-treated grafts.

#### Long-surviving grafts expressing CD40Ig displayed chronic rejection lesions

Long-term graft survival, inhibition of alloantibody and MLR responses, as well as the acceptance of second grafts of donor origin do not necessarily exclude the development of chronic rejection lesions since these phenomena may depend on different immune mechanisms (2). Tissue analysis of long-surviving hearts between days 98 and 260 after transplantation ( $n = 10$ ) revealed the appearance of chronic rejection lesions in 8 of 10 cases (Fig. 7). Vascular lesions were observed in 10 to 60% of the vessels and consisted of intima hyperplasia, leukocyte infiltration of the adventitia, and disorganization of the media (Fig. 7), whereas syngeneic grafted hearts showed normal vessel structure (data not shown). Parenchyma lesions displayed low leukocyte infiltration of the myocardium and focal to moderately diffuse fibrosis (data not shown).

Several leukocyte types have been implicated in the development of chronic rejection (2, 25, 33–35). Among them, CD8<sup>+</sup> cells and eosinophils have been shown to be responsible for chronic rejection in models of CD40-CD40L blockade (23, 25). Immunohistology of long-surviving hearts showed the presence of moderate perivascular infiltration by CD8<sup>+</sup> cells and a lower proportion of CD8 $\beta$ <sup>+</sup> cells (Fig. 7). TCR $\alpha\beta$ <sup>+</sup> cells were present in moderate proportions, and CD161<sup>+</sup> NK cells were less represented (Fig. 7). CD68<sup>+</sup> monocytes/macrophages and CD4<sup>+</sup> cells were the most abundant leukocytes present in the infiltrate (Fig. 7). IFN- $\gamma$ -positive cells were also detected in chronically rejected hearts (Fig. 7). Mast cells were present in higher proportion in the interstitium, but

not around the occluded vessels of hearts expressing CD40Ig compared with syngeneic ( $45 \pm 7$  vs  $15 \pm 4$ , respectively) and native controls (Fig. 7). Eosinophils and IL-4 (data not shown) were undetectable. Neither syngeneic grafts surviving for >100 days after transplantation nor native hearts showed significant staining with any of the above mentioned markers (a low frequency of CD68, CD4<sup>+</sup>, and CD8 $\alpha$ <sup>+</sup> cells was detected) (data not shown).

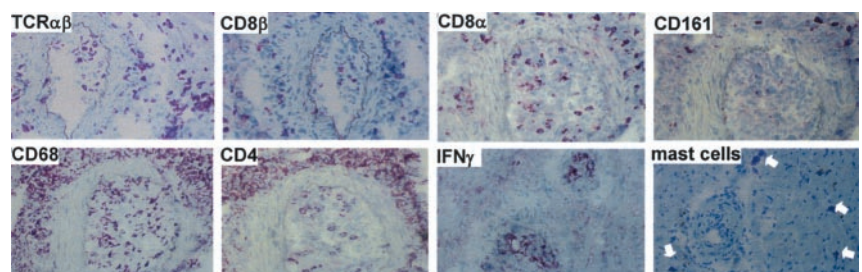
The existence of chronic rejection lesions despite long-term acceptance of first and second grafts of donor origin indicates that not all allogeneic immune responses were abrogated. Although CD8<sup>+</sup> cells were associated with chronically rejected hearts, other leukocytes such as macrophages, CD4<sup>+</sup>, and TCR $\alpha\beta$ <sup>+</sup> cells as well as mast cells were also present in chronically rejected hearts.

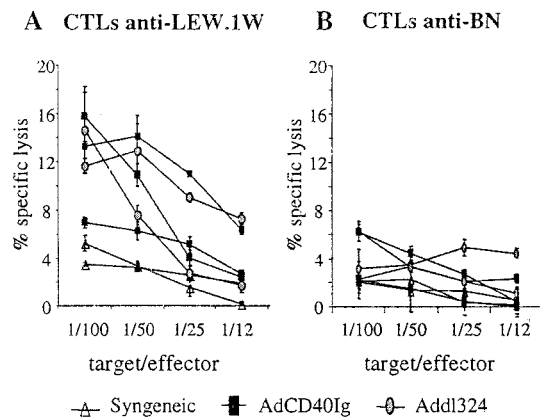
#### Presence of CTL activity in recipients of long-surviving grafts

To further explore the role of CD8<sup>+</sup> cells in chronic rejection of CD40Ig-expressing long-surviving grafts, we analyzed their presence and CTL activity in splenocytes. The percentage of CD8 $\alpha$ <sup>+</sup> cells was augmented among spleen T cells of recipients with long-surviving hearts expressing CD40Ig ( $64 \pm 8\%$ ,  $n = 3$ ) as compared with recipients that had rejected hearts transduced with Addl324 >100 days before ( $31.5 \pm 2\%$ ,  $n = 3$ ), and the total number of splenocytes were comparable (data not shown). Spleen CD4<sup>+</sup> T cells showed a reversed proportion ( $36 \pm 4.5$  and  $65.5 \pm 6$ ) in AdCD40Ig- and Addl324-treated recipients, respectively.

Donor-specific anti-LEW.1W CTL activity in splenocytes from recipients with long-surviving grafts was comparable with that of Addl324-treated control animals that had rejected their grafts

**FIGURE 7.** Histological assessment of leukocytes infiltrating long-surviving grafts. Leukocyte subsets were analyzed with mAbs or May-Grünwald-Giemsa staining (mast cells, arrows) in long-surviving grafts >100 days after transplantation. Results are of one graft representative of eight analyzed with the same markers (objective  $\times 40$ ).





**FIGURE 8.** Antidonor CTL activity in recipients with long-surviving grafts after AdCD40Ig gene transfer. CTL activity of splenocytes from recipients with long-surviving grafts (■), or with grafts rejected >100 days after treatment with Addl324 (○) or syngeneic grafted animals (△) was assayed directly ex vivo against  $^{51}\text{Cr}$ -labeled A, donor LEW.1W or B, third party BN Con A blasts. Results are expressed as mean  $\pm$  SD percentage of specific lysis for individual animals performed in triplicates at different E:T ratios and are representative of two experiments.

>100 days after treatment with Addl324 in four of five cases and decreased in the fifth one (Fig. 8A, and data not shown). Syngeneic control animals showed low nonspecific anti-LEW.1W CTL activity (Fig. 8A). All animals of the three groups showed low anti-third party (BN) (Fig. 8B) or antisynthetic (LEW.1A) (data not shown) CTL activity.

These data indicate that antidonor CTL activity was present in splenocytes of CD40Ig-treated recipients, but the increase in CD8 $\alpha^+$  T cells suggests that the overall CTL activity of the CD8 $\alpha^+$  population is partially decreased in CD40Ig-treated recipients.

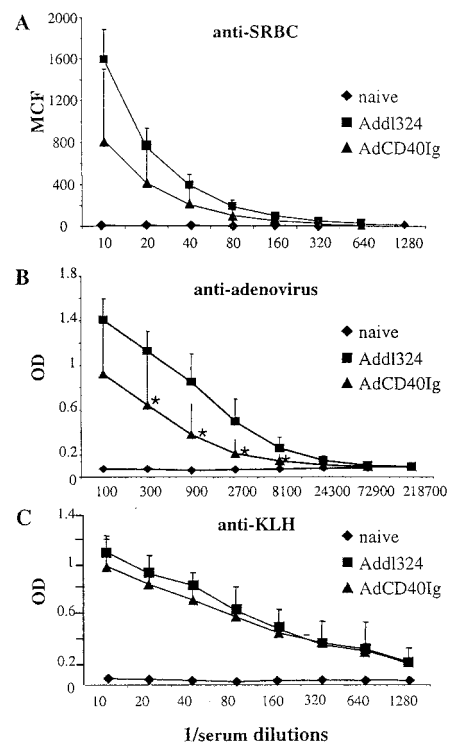
#### Immune responses against cognate Ags in recipients of AdCD40Ig-treated grafts

To evaluate whether recipients that showed inhibition of allogeneic immune responses after CD40Ig expression also had suppressed reactivity against cognate Ags, we injected SRBC or KLH at day 0 or >100 after transplantation, respectively, and analyzed their specific immune response as well as the presence of anti-adenovirus Abs at early and late time points.

Levels of IgG (Fig. 9A) and IgM (data not shown) anti-SRBC Abs were decreased in the group of rats with AdCD40Ig-treated grafts compared with Addl324 controls, but this decrease was not significant despite three of five animals showing inhibited responses. Reduction of anti-SRBC Ab levels in these animals did not correlate with higher serum concentrations of CD40Ig (data not shown).

Titers of IgG anti-adenovirus Abs were partially, but significantly inhibited at day 17 (Fig. 9B) and >200 days ( $n = 5$ , data not shown) after gene transfer in rats with AdCD40Ig-treated grafts compared with Addl324 controls. IgM anti-adenovirus Abs were not significantly inhibited at day 17 and >200 days after gene transfer in the AdCD40Ig vs Addl324 group ( $n = 5$  in each group, data not shown).

IgG (Fig. 9C) and IgM (data not shown) Abs against KLH injected >100 days after gene transfer were similar in all recipients of AdCD40Ig-treated grafts compared with Addl324 controls. Furthermore, proliferative responses of cells from draining lymph nodes against KLH were identical in both groups of animals (data not shown).



**FIGURE 9.** Systemic T cell-dependent Ab responses against cognate Ags in recipients of AdCD40Ig-transduced grafts. A, IgG anti-SRBC Abs. Rats grafted with hearts transduced with Addl324 ( $n = 4$ ) or AdCD40Ig ( $n = 5$ ) were immunized with SRBC at the day of transplantation. Levels of IgG anti-SRBC Abs were analyzed in serially diluted heat-inactivated serum by cytofluorometry at day 17 after transplantation. Results are expressed as mean channel fluorescence (MCF  $\pm$  SD). B, IgG anti-adenovirus Abs. Anti-adenovirus Abs were measured in naive animals or 17 days after gene transfer with  $5 \times 10^{11}$  adenoviral particles of AdCD40Ig ( $n = 5$ ) or Addl324 ( $n = 5$ ). Results are expressed as mean OD  $\pm$  SD. C, IgG anti-KLH Abs. KLH (50  $\mu\text{g}$  emulsified in 200  $\mu\text{l}$  CFA) was injected into the footpad of animals >100 days after transplantation with hearts transduced with Addl324 ( $n = 2$ ) or AdCD40Ig ( $n = 4$ ), and levels of anti-KLH Abs in sera were analyzed by ELISA 9 days later. Results are expressed as mean OD  $\pm$  SD. Naive animals were not immunized against SRBC, adenoviruses, or KLH ( $n = 3$ ). \*,  $p < 0.05$  as compared with Addl324-treated animals.

Therefore, recipients of grafts expressing CD40Ig showed systemic immune responses against cognate Ags that were partially conserved when initiated at early time points and normal thereafter, despite complete inhibition of early and late anti-donor Ab and proliferative responses.

## Discussion

Blockade of CD40-CD40L interactions through gene transfer-mediated expression of CD40Ig resulted in long-term acceptance of allogeneic hearts in rats. Despite profound inhibition of alloantibody production and allogeneic proliferation as well as acceptance of second grafts of donor but not of third party origin, long-surviving grafts showed chronic rejection lesions. Recipients of CD40Ig gene transfer displayed conserved immune responses against cognate and third party alloantigens, indicating maintenance of a variety of immune mechanisms implicated in immune responses against environmental Ags.

In mice and primates, anti-CD40L mAb therapy alone (with multiple injections) has resulted in long-term acceptance of vascularized organs and islet transplants, but not of skin (10, 13–16, 18, 36). Our study in rats using a single gene transfer of CD40Ig into the graft or

at distant sites resulted, in both cases, in long-term cardiac graft survival. Differential effects of anti-CD40L mAb and CD40Ig therapy may depend on different affinities and timing of action since Abs have a limited bioavailability, whereas CD40Ig is continuously produced. The fact that CD40Ig was being secreted into the circulation and acting both at the graft site as well as in secondary lymphoid organs in both situations does not enable conclusions to be made concerning whether inhibition of the immune responses was occurring predominantly within the graft or in the lymphoid organs. It is likely that the effects of CD40Ig were present at both sites since blockade of CD40-CD40L interactions inhibits Ag presentation (10), occurring mainly in secondary lymphoid organs, but it has also been shown to inhibit ongoing acute graft rejection (10, 19), indicating that blockade of CD40-CD40L can also inhibit the effector phase (in the graft) of immune responses. These results and those previously published on adenovirus-mediated CTLA4Ig expression (which also resulted in long-term graft acceptance) (8, 9) indicate that although gene transfer is performed in the graft itself, production and secretion of molecules can give rise to high circulating levels with actions at distant sites. This positive aspect of gene transfer is counterbalanced by potential systemic immunosuppressive actions, which were only partially observed for CD40Ig (see below), and may demand the use of inducible promoters to control transgene expression during time.

Our results show that CD40Ig had a moderate impact on local mechanisms since it resulted in a partial reduction in leukocyte infiltration. Nevertheless, the frequency of iNOS-, CD25-, HO-1-, and IFN- $\gamma$ -expressing cells detected by immunohistology within the graft was comparable with controls, although we cannot exclude that the total amount of these inflammatory mediators could be decreased if evaluated by more quantitative techniques. Our results are in agreement with previous publications in mice and primates showing that animals treated with anti-CD40L mAb alone (12, 18, 19, 36) or CD40L-deficient recipients (24) displayed no or only a moderate (50%) reduction in leukocyte infiltration. Similarly, cytokines showed no or a moderate decrease in the absence of CD40-CD40L interactions (12, 18, 24, 36). For both, leukocytes and cytokines, combinations of anti-CD40L mAbs and CTLA4Ig (18), donor-specific blood transfusion (12), or anti-CD45RB (36) resulted in a marked inhibition.

Proliferative responses against donor Ags at early and late time points after transplantation were strongly inhibited with splenocytes and partially inhibited with T cells as responder cells. The preferential inhibition when splenocytes were used as responders may depend on the absence of efficient Ag presentation and/or the production of inhibitory molecules by APCs (37). Alternatively, and as already demonstrated in anti-CD40L-treated mice in which the generation of suppressive T cells performing linked suppression was described (15), triggering of regulatory T cells responsible for graft acceptance may require indirect alloantigen presentation by recipient APCs. Previous results have also shown donor-specific hyporesponsiveness in MLRs of primates treated with anti-CD40L mAbs (19, 20) or mice deficient for CD40L (24) harboring long-surviving organs.

IgG alloantibody responses were profoundly inhibited in rats expressing CD40Ig, a finding common to some (24, 25, 36, 38, 39), but not all (19) transplantation models by blocking or in the absence of CD40-CD40L interactions. Ab responses against T cell-dependent cognate Ags were partially conserved at early time points and completely normal in recipients with long-surviving grafts, and this may be explained by the higher serum concentration of CD40Ig at early compared with late time points. Anti-adenovirus immune responses have been described as being partially (40–42) or completely (43, 44) blunted by blocking or in the absence of CD40-CD40L interactions. This is consistent with our

results showing partial inhibition of early (day 17) and late (>200 days) anti-adenovirus IgG Ab responses and with the long-term expression of CD40Ig (>5  $\mu\text{g}/\text{ml}$  160 days after gene transfer). Altogether, these results are in agreement with those reported by Gray et al. (45), in which *in vivo* administration of the same CD40Ig molecule as that used in our study resulted in partial (50–60%) inhibition of Ab responses against a cognate Ag and no inhibition of germinal center formation. Differences in anti-allogeneic and cognate immune responses may depend on requirements for different costimulatory signals as well as on the presence of allospecific regulatory cells. Although we cannot formally exclude the possibility that high circulating levels of CD40Ig could have some nonspecific inhibitory effects on Ab responses, previously published data on the inhibition of Ab production in the absence of CD40-CD40L interactions and the lack of an alloantibody decrease in animals treated with a recombinant adenovirus expressing high levels of the TNF-related activation-induced cytokine receptor fused to the same Fc fragment (data not shown) argue against this possibility. The conservation of systemic immune responses against cognate Ags is an important point in favor of blocking CD40-CD40L interactions vs blockade of B7-CD28 by CTLA4Ig gene transfer in which anticognate Ag responses were completely blocked not only at early, but also at late time points after gene transfer (9).

Recipients of AdCD40Ig-transduced grafts showed evidence of donor-specific mechanisms of unresponsiveness since: 1) they accepted second hearts of donor origin and rejected third party grafts; 2) showed suppressed humoral and cellular donor-specific immune responses. These results fit with the definition of operational tolerance (21, 22), a state of long-term graft acceptance with acceptance of second donor-derived grafts in the absence of chronic immunosuppression with anticognate and third party immune responses being conserved. Nevertheless, the presence of chronic rejection demonstrates that these animals did not develop complete transplantation tolerance. Similar data have been recently published using CD40L-deficient mice in whom chronic rejection appeared despite donor-specific acceptance of second grafts (24). Chronic rejection lesions were also described in long-surviving grafts after treatment with anti-CD40L mAbs (18, 23, 25).

Some acute rejection models dependent on CD8<sup>+</sup> T cells are not blocked by anti-CD40L mAbs as efficiently as those dependent on CD4<sup>+</sup> T cells (15–17). Nevertheless, the role of CD8<sup>+</sup> cells in chronic rejection following interruption of CD40-CD40L interactions has resulted in partially contradictory results (23, 25). In a mouse aortic chronic rejection model, depletion of CD8<sup>+</sup> cells in anti-CD40L-treated mice led to partial, but significant prevention of chronic rejection (23), whereas the same treatment in CD40-deficient recipients did not prevent chronic rejection (25). Our results show the presence of CD8 $\alpha$  and CD8 $\beta$ <sup>+</sup> cells in vessels of chronically rejected organs, a large increase in CD8 $\alpha$ <sup>+</sup> T cells within splenocytes, and the presence of antidonor CTLs, suggesting that CD8<sup>+</sup> cells could be implicated in chronic rejection of CD40Ig-treated grafts. In aortic (25) and cardiac (34) transplantation models, depletion of CD8<sup>+</sup> cells resulted in infiltration by eosinophils through production of IL-4 and IL-5. IFN- $\gamma$  produced by CD8<sup>+</sup> T cells was responsible for inhibiting eosinophil recruitment within the grafts (34), indicating that eosinophils may play a significant role in rejection of vascularized models following CD8<sup>+</sup> cell depletion, but not when CD8<sup>+</sup> cells are present (34). This is in agreement with our results showing the presence of CD8<sup>+</sup> and IFN- $\gamma$ <sup>+</sup> cells and the absence of eosinophils in chronically rejected grafts. Interestingly, increased proportions of mast cells were observed in CD40Ig-treated chronically rejected hearts, as previously described in another model of chronic rejection in

rats (35). Nevertheless, other leukocytes, such as macrophages and CD4<sup>+</sup> cells, also infiltrated the vascular wall and the periphery of occluded vessels of CD40Ig-treated grafts. Therefore, additional experiments, which are beyond the scope of this study, are needed to elucidate the role of each of these cell types and certain molecules (such as IFN- $\gamma$ ) in this model of chronic rejection. Although antiviral immune responses contribute to the development of chronic rejection (2), several reasons argue against the possibility that anti-adenovirus immune responses in our model contribute to this process. First, one of two long-surviving heart grafts, after liver transduction with AdCD40Ig (and therefore in the absence of local anti-adenovirus immune responses), showed signs of chronic rejection. Second, long-surviving heart grafts, after transduction with an adenovirus coding for CTLA4Ig, did not show signs of chronic rejection (9). Third, chronic rejection has been described following treatment with anti-CD40L Abs or in CD40L knockout animals (18, 23–25). Fourth, anti-adenovirus immune responses were partially blocked by CD40Ig, at least at the level of CD4<sup>+</sup> T-dependent IgG production, therefore reducing the likelihood of chronic rejection as a consequence of anti-adenoviral immune responses.

In conclusion, prolonged expression of CD40Ig by gene transfer resulted in efficient blockade of acute rejection with the development of donor-specific hyporesponsiveness and the presence of conserved immune responses against cognate Ags. The development of chronic rejection in long-surviving grafts despite long-term continuous inhibition of CD40-CD40L interactions indicates that this therapy needs to be complemented by other therapeutic strategies to obtain true donor Ag-specific tolerance.

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