Effect of Administration of CTLA4-Ig as Protein or cDNA on Corneal Allograft Survival

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Purpose. To examine the role of the CD28–CD80–CD86 pathway of T-lymphocyte costimulation in corneal allograft rejection and the effect of blockade of that pathway on graft survival.

Methods. Kinetics of CD80 and CD86 expression in the cornea and draining lymph nodes were examined by RT-PCR and immunohistochemistry in untreated allograft recipients in a high-responder rat model. The effect of blockade of CD28-mediated costimulation was first examined by ex vivo incubation of excised Brown Norway rat donor cornea with the inhibitory protein CTLA4-Ig or an adenovirus vector (AdCTLA) expressing CTLA4-Ig, before grafting into Lewis rat recipients. A second group of graft recipients received systemic posttransplantation treatment with either CTLA4-Ig or AdCTLA.

Results. Expression of CD80 mRNA was increased in both donor and recipient cornea 16 hours after transplantation, whereas CD86 was detected constitutively, with no significant early increase. Immunohistochemistry on day 5 after transplantation demonstrated major histocompatibility complex (MHC) class II expression, no CD80, and only a trace of CD86 in corneal allografts. In lymph nodes strong MHC class II, weak CD80, and moderate CD86 expression was noted. Both donor cornea and recipient treatment with CTLA4-Ig resulted in prolonged allograft survival. AdCTLA was found to induce sustained secretion of bioactive CTLA4-Ig from corneas infected ex vivo. Survival of corneal allografts incubated with AdCTLA was marginally prolonged, and systemic treatment with AdCTLA significantly prolonged survival.

Conclusions. Protein- or gene-based administration of CTLA4-Ig prolongs allograft survival by treatment of either the recipient or the donor tissue ex vivo before grafting. (*Invest Ophthalmol Vis Sci.* 2002;43:1095–1103)

Transplant rejection is primarily dependent on activation of alloreactive T lymphocytes and is the leading cause of corneal allograft failure.[1,2] A number of signals have been identified that lead to T-cell activation. The first signal, which gives specificity to the immune response, is given by the interaction of alloantigen–major histocompatibility complex (MHC) expressed on antigen-presenting cells (APCs) with the T-cell receptor–CD3 complex expressed on the T cell.[3] However full activation of the T cell requires a second category of costimulatory signals. This can be provided by signaling through CD28 on the T cell after interaction with CD80 (B7-1) and CD86 (B7-2) on the APC surface.[4] A homologous T-cell surface molecule CTLA-4 (CD152) binds B7 molecules with higher affinity than CD28, but transduces negative regulatory signals and arrests cell cycle progression.[5,6] In the absence of a positive costimulatory signal, the IL-2 gene is not transcribed. T cells fail to proliferate or respond and may be rendered anergic to further antigenic challenge.[7]

CTLA4-Ig is a recombinant form of CTLA4 that acts as a competitive inhibitor of CD28-mediated costimulation.[8] This fusion protein comprises the extracellular domains of the CTLA4 receptor linked to an IgG heavy chain. The importance of the CD28 costimulatory pathway in vivo has been illustrated by the significant effect on graft survival of CTLA4-Ig treatment in recipients of fully MHC-mismatched rat cardiac,[9] rena1,[10] and lung[11] allografts and even in mouse xenografts.[12] In the absence of systemic treatment of the graft recipient, ex vivo coating of donor pancreatic islet cells with murine CTLA4-Fc fusion protein has been reported to promote allograft tolerance.[13] CTLA4-Ig has been shown to prolong survival in both mouse and rabbit models of corneal transplantation.[14,15] In this study, we examined the early kinetics of CD80 and CD86 expression in the cornea and draining lymph nodes after corneal transplantation. We then demonstrated the variable effects of blockade of CD28 costimulation, by local and systemic treatment with either CTLA4-Ig or a recombinant virus expressing this protein.

Methods

Animals and Corneal Transplantation

In all experiments adult female Lewis (LEW, RT1^l) strain rats served as recipients and Brown Norway (BN, RT1^n) strain rats as donors. Animals were obtained from Harlan Olac (Bicester, UK). Transplantation of 3.5-mm diameter donor cornea into a 3.0-mm diameter recipient corneal bed was performed as previously described.[16] Sutures were removed on day 7 after transplantation. Grafts were examined daily and the day of rejection defined as previously described.[17] The ARVO Statement for the Use of Animals in Ophthalmic and Vision Research was adhered to at all times.

Quantitation of mRNA

Donor and the surrounding recipient corneas were excised, dissected, snap frozen in liquid nitrogen and stored at −70°C, with two animals examined at each time point. Normal nontransplanted cornea from LEW rats acted as the control (designated day 0). The superficial cervical lymph nodes were excised from graft recipients through a small incision made in the neck skin on the ipsilateral and contralateral sides, snap frozen in liquid nitrogen, and stored at −70°C. Competitive RT-PCR was performed as described by King et al.,[18] using the following primers made by MWG-Biotech (Milton Keynes, UK). Hypoxanthine phosphoribosyltransferase (HPRT) sense, TCC CAG CGT CGT GAT TAG TG; HPRT antisense, GGC TTT CCA CTT TCG CTG A; CD80 sense, GGC ATT GCT GTC CTG TGA TTA C; CD80 antisense, ACT CAG

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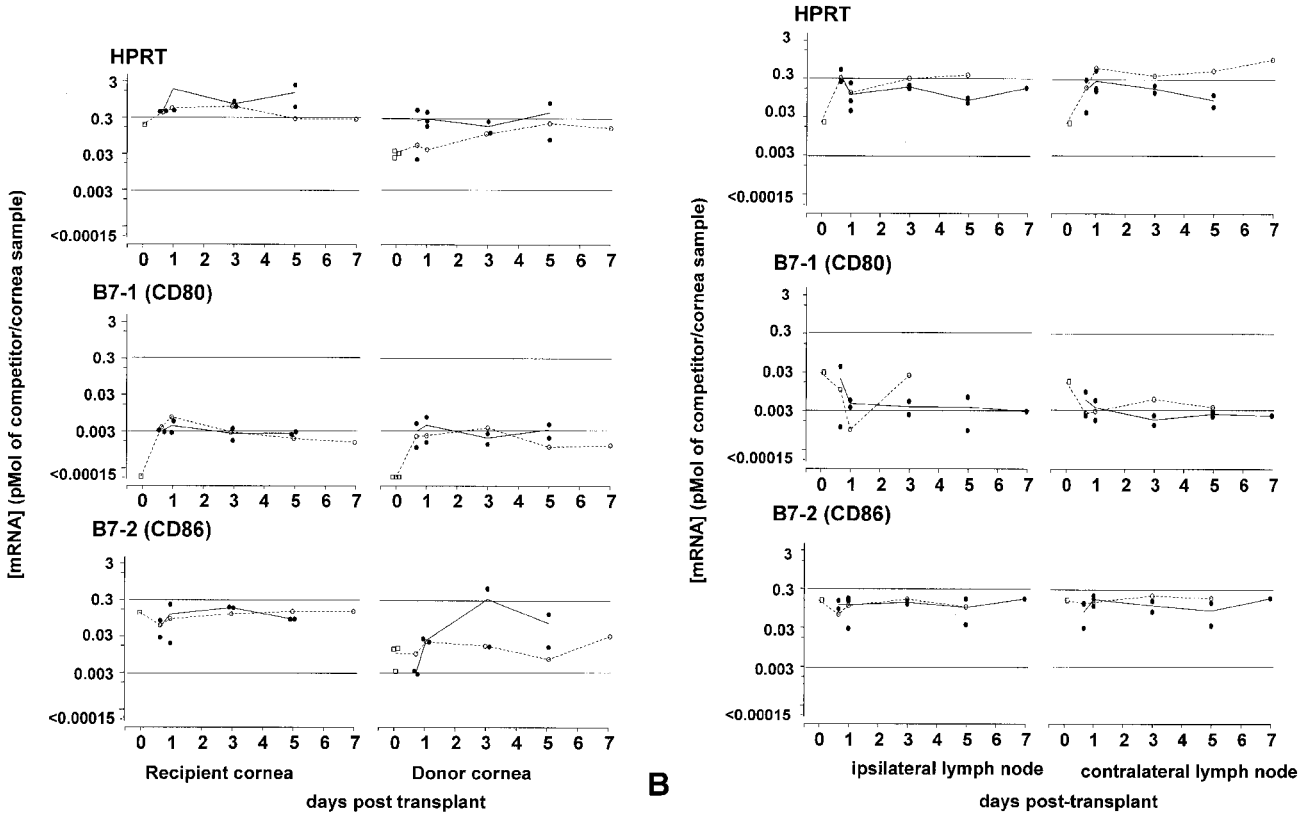


FIGURE 1. Expression of CD80 and CD86 mRNA in cornea and draining lymph nodes. Messenger RNA is expressed as picomoles of the competitor sample at the point of equivalence per corneal sample. CD80 mRNA was not detected in normal donor or recipient cornea (□). Levels of expression were increased, albeit remaining at a low level, 16 hours after grafting in both donor and recipient corneas with similar expression seen at days 3 and 5 (○, syngeneic graft; ●, allogeneic graft). CD86 mRNA was constitutively expressed in both central and peripheral cornea, with no detectable change at any time point after transplantation (**A**). CD80 and CD86 mRNA were constitutively expressed in draining lymph nodes, with no detectable difference after transplantation (**B**).

TTA TGT TGG GGG TAG G; CD86 sense, TCT CAG ATG CTG TTC CTG TG; and CD86 antisense, GTA GGT TTC GGG TAT CCT TG.

Deletional competitor plasmids were generated as previously described,[19,20] using the appropriate deletion primers followed by TA cloning (Invitrogen, Groningen, The Netherlands). The CD80 sense deletion primer sequence was GGC ATT GCT GTC CTG TGA TTA CAG TAC AAG AAC CGG AC and the CD86 sense deletion primer was TCT CAG ATG CTG TTC CTG TGC GGT TCT GTA CGA ACA. For each mRNA, the point of equivalence on competitive RT-PCR was determined (i.e., the point at which the *Taq* polymerase amplified an equivalent amount of both the wild-type product and the competitor plasmid product).[18]

Immunohistochemistry

Immunohistochemistry was performed on 5-μm-thick cryostat sections of frozen, excised, whole cornea, with two animals examined at each time point. After acetone fixation, mouse monoclonal antibodies specific for rat MHC class II (MCA95, diluted 1:5, Serotec, Oxford, UK), CD80, and CD86 (clones 3H5 and 24F, respectively, both diluted 1:100; gifts from Hideo Yagita, Juntendo University School of Medicine, Japan) were used as primary antibodies. Biotinylated goat anti-mouse antibody (Dako, Glostrup, Denmark) was used as a secondary antibody at 1:100 dilution in 3% rat serum (Sigma, Poole, UK), followed by streptavidin-peroxidase complex (Dako). Sections were then incubated in diaminobenzidine chromogen (Sigma) and counterstained with hematoxylin. Appropriate positive and negative controls were used for each antibody.

Production and Quantitation of CTLA4-Ig

CTLA4-Ig, comprising mouse CTLA4 linked to a human Ig domain, was purified by protein A chromatography from the supernatant of transfected J588L cells to 200 μg/mL, as previously described.[21] Levels of CTLA4-Ig fusion protein were measured by sandwich ELISA[22] as follows, in brief. ELISA plates (NalgeNunc International, Roskilde, Denmark) were coated with a rabbit anti-human IgG γ-chain–specific antibody diluted at 1:500 (Dako) in 0.1 M $NaHCO_3$ (pH 9.6) and incubated for 1 hour at 37°C in 5% CO_2. Plates were washed three times with PBS and 0.1% Tween after each step. The plate was then blocked with 2% milk for 30 minutes, before adding the standards (human IgG_1; Sigma) or samples in triplicate to each well, and incubated for 1 hour at 37°C. The secondary antibody was a biotinylated goat anti-human IgG γ-chain–specific antibody used at a dilution of 1:5000 (Dako), after which a streptavidin-horseradish peroxidase (HRP)–conjugated enzyme (Dako) was added at a concentration of 1:1000 and the plate reincubated for 30 minutes at 37°C. A 3,3′,5′5-tetramethylbenzidine (TMB) single-solution substrate (Cambridge Bioscience, Cambridge, UK) was then added and the reaction allowed to develop for 15 minutes before it was stopped by the addition of 50 μL 5% H_2SO_4 to all wells. Plates were read at 450 nm, with a microtiter plate reader (Titretek Multiscan MCC 340; Laboratory Systems, Basingstoke, UK). Functional activity was quantified in one-way BN-LEW mixed-lymphocyte reactions (MLRs). These were performed as described,[23] using 5×10^5/mL irradiated BN splenocytes as stimulators and 5×10^5/mL LEW lymph node cells as responders in 200 μL medium/well.

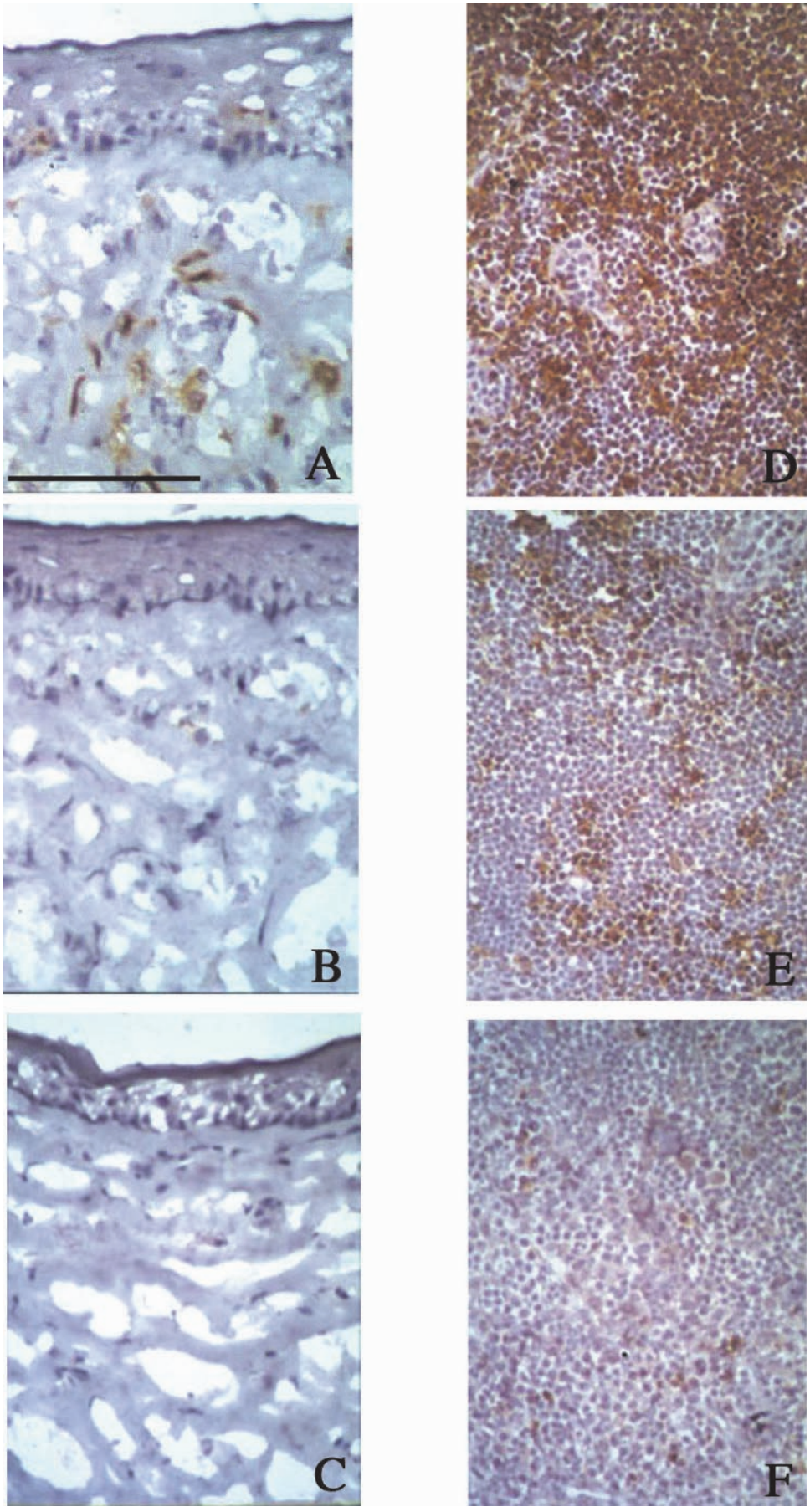


FIGURE 2. Expression of APC markers 5 days after transplantation of allogeneic cornea. Immunoperoxidase staining of donor cornea: (**A**) moderate (++) staining for MHC class II, (**B**) no staining for CD80, (**C**) no staining for CD86. Immunoperoxidase staining of draining lymph nodes: (**D**) strong (+++) staining for MHC class II, (**E**) moderate staining for CD86, (**F**) weak (+) staining for CD80. Scale bar, 50 μm.

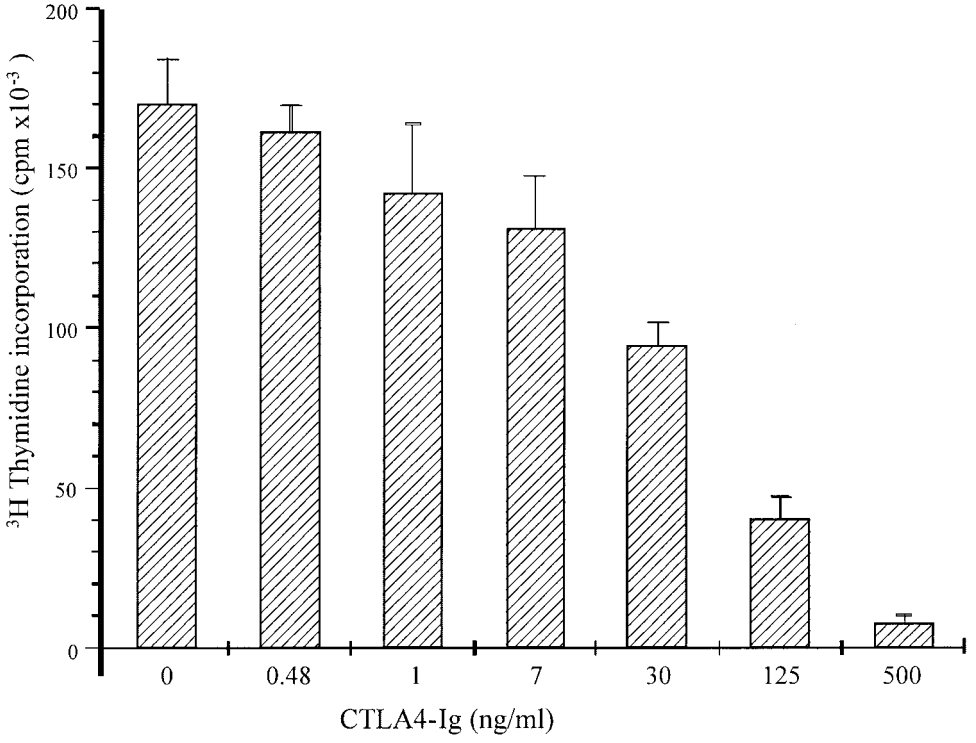


FIGURE 3. Functional activity of purified CTLA4-Ig was tested by adding it in a one-way BN-LEW MLR. T-cell proliferation was inhibited in a dose-dependent manner. Results are expressed as the mean ± SD of triplicate cultures.

Administration of CTLA4-Ig

The effect of donor cornea treatment with CTLA4-Ig before transplantation was examined by incubating excised BN corneas (3.5 mm diameter) for 18 hours at 4°C in culture medium containing CTLA4-Ig (0.2, 2, or 20 μg/mL). Epithelium was gently removed from the central 4.0 mm of donor corneas before incubation with CTLA4-Ig, using a size 21 surgical blade (Swan-Morton, Sheffield, UK), because it was found in preliminary experiments that overgrowth of the endothelial surface by epithelium occurred during the period of culture. Corneas were washed before orthotopic transplantation.

To examine the effect of systemic treatment with CTLA4-Ig, protein was administered to graft recipients by intraperitoneal injection of 100 μg (1.5 mL) on day 2 after transplantation and 50 μg daily until day 10 (total dose, 500 μg). Isotype-matched human IgG (Sigma) was used as a negative control in donor cornea and systemic treatments.

Recombinant Adenoviruses

Recombinant E1-deleted type 5 adenovirus vectors AdCTLA and Ad0 were used in experiments, both kindly provided by Matthew Wood, University of Oxford. AdCTLA incorporates cDNA sequences encoding the extracellular portion of mouse CTLA4 and the hinge, CH_2, and CH_3 domains of human IgG, placed under transcriptional control of the Rous sarcoma virus (RSV) promoter.[22] Ad0 is a null vector control with an E1 deletion but no cDNA insert. Viruses were propagated and purified and the titer quantified in plaque-forming units (PFU), as previously described.[24]

Determination of Optimum AdCTLA Concentration for Transduction of Corneal Endothelium and Kinetics of CTLA4-Ig Secretion

BN corneas were incubated in 200 μL 2% fetal calf serum (FCS) in MEM containing various concentrations of AdCTLA or Ad0 for 3 hours at 37°C in 5% CO_2. After incubation, specimens were washed three times with Hanks' balanced salt solution (HBSS). The cornea samples were then maintained in MEM supplemented with 10% FCS, 2 mM L-glutamine, 100 U/mL penicillin, and 0.1 mg/mL streptomycin at 37°C and 5% CO_2. Culture medium was changed every 2 days.

Adenovirus-Mediated Gene Transfer

The effect of ex vivo gene transfer to donor cornea before transplantation was examined by incubation of de-epithelialized donor corneas for 3 hours at 37°C in culture medium containing 5×10^7 PFU/mL AdCTLA. Corneas were then washed twice and incubated in virus-free culture medium before transplantation. Administration of virus was by injection of 100 μL 1×10^9 PFU/mL AdCTLA or Ad0 into a tail vein on the first day after transplantation.

Statistical Analysis

Graft survival data were analyzed by Kaplan-Meier test and the Mann-Whitney unpaired nonparametric test. Differences between treatment groups were considered significant if $P < 0.05$.

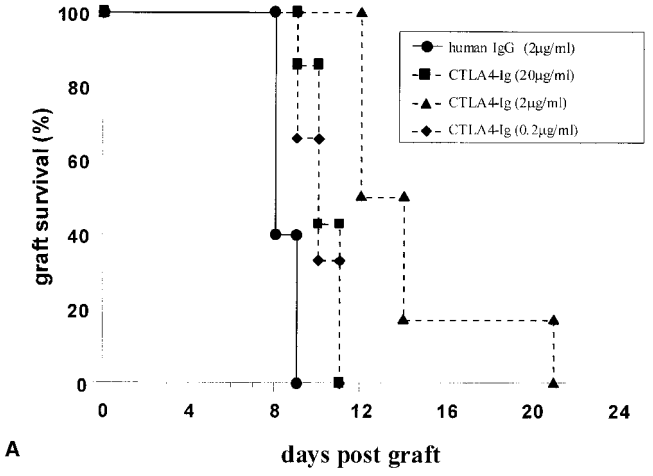
RESULTS

Expression of CD80 and CD86 mRNA in Cornea and Draining Lymph Nodes

CD80 mRNA was not detected in normal donor or recipient cornea. Levels of expression were increased, albeit remaining at a low level, 16 hours after grafting in both donor and recipient corneas with similar expression seen at days 3 and 5. CD86 mRNA was constitutively expressed in both central and peripheral cornea, with no detectable change at any time point after transplantation (Fig. 1A). CD80 and CD86 mRNA were constitutively expressed in draining lymph nodes, with no detectable difference after transplantation (Fig. 1B).

Immunohistochemical Staining of Corneas and Lymph Nodes

There was weak staining in corneas for MHC class II but no staining for CD80 or CD86 on day 1 after transplantation and moderate staining for MHC class II with trace staining of CD86 and no staining for CD80 on day 5. Draining lymph nodes were strongly positive only for MHC class II on day 1 and moderately



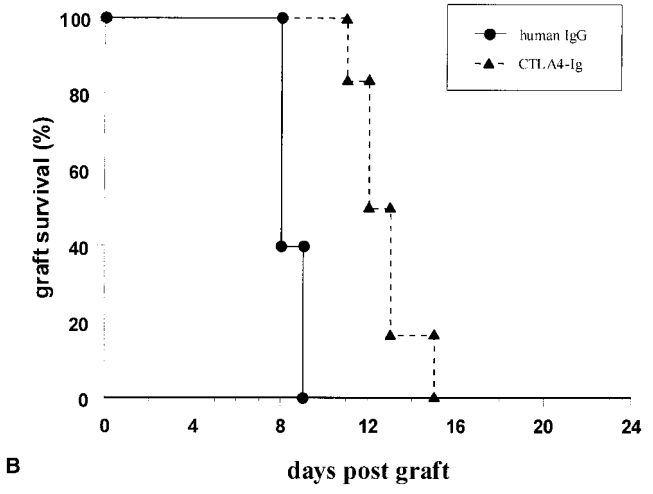


FIGURE 4. Corneal allograft survival after administration of CTLA4-Ig. (**A**) Ex vivo treatment of donor cornea with CTLA4-Ig. Donor corneas were incubated with CTLA4-Ig or human IgG before transplantation and assessed daily for signs of rejection. The most significant increase in allograft survival compared with that of control corneas ($n = 5$; MST = 8 days) was found with 2 μg/mL CTLA4-Ig ($n = 6$; MST = 14 days). Corneas incubated in 0.2 μg/mL or 20 μg/mL CTLA4-Ig demonstrated only moderately prolonged survival ($n = 6$; MST = 10 days, both doses). (**B**) Systemic CTLA4-Ig treatment of graft recipient. Prolonged allograft survival was found in the group treated with intraperitoneal CTLA4-Ig ($n = 6$; MST = 13 days,) compared with isotype-matched human IgG control grafts ($n = 5$; MST = 8 days). Allografts in untreated recipients consistently underwent rejection at day 9.

positive for CD86 and weakly positive for CD80 on day 5, with no change in class II expression (Fig. 2).

Functional Activity of Purified CTLA4-Ig

As expected, CTLA4-Ig significantly decreased [^{3}H]thymidine incorporation by responder cells on day 3 after incubation in a dose-dependent manner (Fig. 3). Concentrations as low as 30 ng/mL reduced proliferation to 50% of that seen in untreated control cultures, confirming the functional activity of CTLA4-Ig.

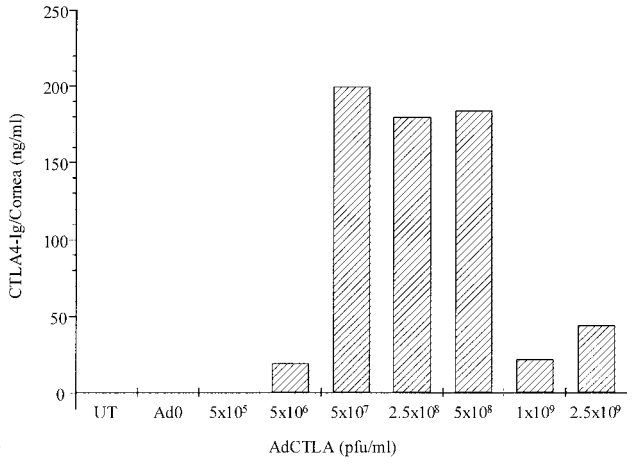
Ex Vivo Donor Cornea or In Vivo Systemic Treatment with CTLA4-Ig

To examine the effect of local treatment, corneas were incubated in various concentrations of CTLA4-Ig overnight. Those incubated in 2 μg/mL CTLA4-Ig had the longest survival as allografts ($n = 6$, median survival time [MST] = 14 days, $P = 0.0043$) compared with control grafts ($n = 5$, MST = 8 days) and those incubated in 0.2 μg ($n = 6$, MST = 10, $P = 0.0173$) or 20 μg/mL ($n = 6$, MST = 10, $P = 0.0051$; Fig. 4A).

In vivo administration of protein to LEW recipients of unmodified BN allografts comprised daily intraperitoneal injections after transplantation, according to a treatment regimen identical with that reported by Turka et al.[25] to prolong survival of BN hearts in LEW recipients (Fig. 4B). Prolonged corneal allograft survival was found in the group treated with CTLA4-Ig ($n = 6$, MST = 13, $P = 0.0043$) compared with control grafts ($n = 5$, MST = 8).

Kinetics of CTLA4-Ig Secretion by AdCTLA-Transduced Corneas

We have previously shown that adenovirus-mediated gene transfer to excised whole corneas is restricted to the endothe-



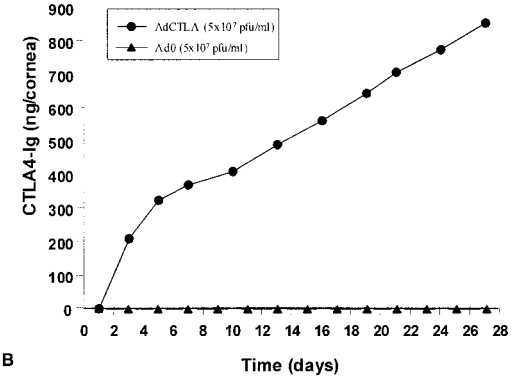


FIGURE 5. Transduction of donor cornea with adenovirus vector. (**A**) The optimal concentration of AdCTLA for gene transfer was determined by ELISA measurement of the concentration of CTLA4-Ig secreted by transduced corneas 3 days after infection. Transduction efficiency reached threshold levels at 5×10^7 PFU/mL AdCTLA. No protein was detected in supernatant from any untransduced or Ad0-transduced corneas. (**B**) To assess the time course of CTLA4-Ig production, corneas were transduced with 5×10^7 PFU/mL AdCTLA or Ad0 for 3 hours, washed, and maintained in ex vivo culture for 27 days. Culture supernatants were collected every 2 days and secreted CTLA4-Ig measured by ELISA. Cumulative CTLA4-Ig production continued to 27 days after transduction, with maximal production evident in the first 7 days. Data are cumulative amounts of CTLA4-Ig produced by one representative 3.5-mm diameter BN cornea transduced with AdCTLA and one transduced with Ad0.

lium.[24] To identify optimum virus concentrations for production of CTLA4-Ig, secreted CTLA4-Ig in corneal culture supernatant was quantified by ELISA 3 days after corneal incubation with AdCTLA (Fig. 5A). BN corneas transduced with 5×10^7 PFU/mL AdCTLA and maintained in ex vivo culture were found to secrete maximal levels of CTLA4-Ig in the first 7 days, and cumulative production continued to 27 days, when the experiment was terminated (Fig. 5B). Adenovirus-derived CTLA4-Ig was demonstrated in an MLR to reduce responder T-cell proliferation in a dose-dependent manner (data not shown).

Effect of Intraperitoneal and Topical Adenovirus-Mediated Gene Transfer of CTLA4-Ig on Corneal Allograft Survival

Gene delivery using an adenovirus vector, either directly to the donor cornea before transplantation or injected intravenously, was compared. BN donor rat corneas transduced ex vivo with 5×10^7 PFU/mL AdCTLA survived marginally longer ($n = 10$, MST = 10 days) compared with allografts transduced with Ad0 ($n = 5$, MST = 9 days, $P = 0.013$; Fig. 6A). Although statistically significant, this indicates that the effect of topical gene transfer of CTLA4-Ig to the corneal endothelium is not sufficient alone to meaningfully prolong allograft survival, at least in the high-responder BN–LEW donor–recipient strain combination. Significantly longer survival was seen in the group treated with 1×10^9 PFU/mL intravenous AdCTLA ($n = 5$; MST = 18) than in the Ad0 group ($n = 5$, MST = 9 days, $P = 0.008$; Fig. 6B). All graft recipients in the AdCTLA group demonstrated significant prolongation of allograft survival, with one graft surviving more than 100 days.

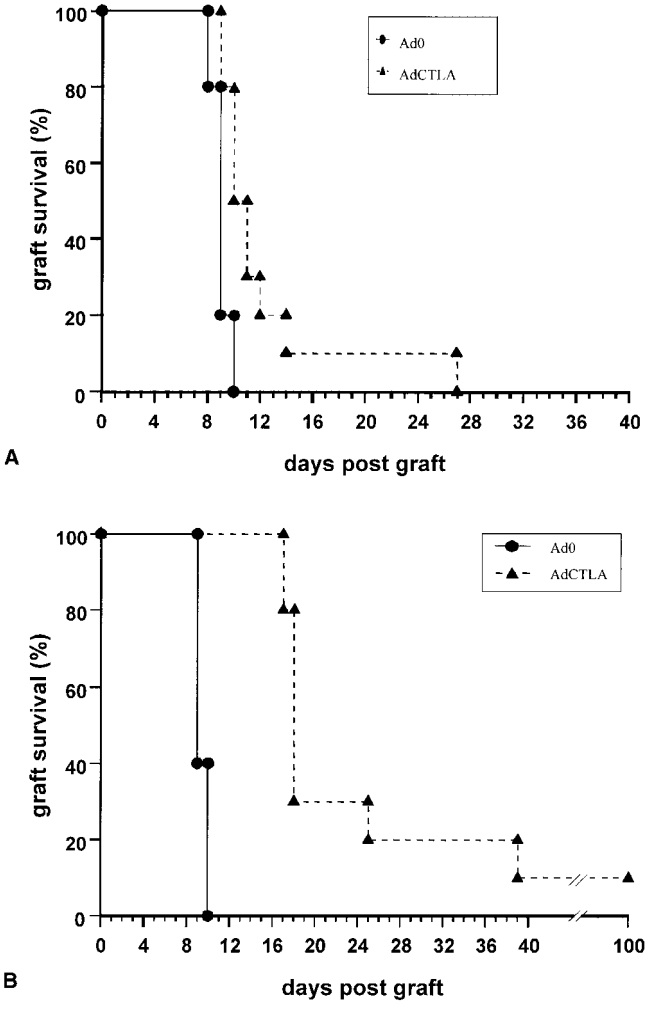


FIGURE 6. Corneal allograft survival after adenovirus-mediated gene transfer of CTLA4-Ig. (**A**) Ex vivo transduction of donor cornea with 5×10^7 PFU/mL AdCTLA before transplantation resulted in longer survival than in Ad0-transduced grafts. (**B**) Greater prolongation of graft survival was found after intravenous injection of AdCTLA on day 1 after transplantation. MST in Ad0-treated recipients was identical with that in unmodified control animals (data not shown).

CTLA4-Ig Concentration in Serum after Intravenous AdCTLA

After intravenous injection of 1×10^9 PFU/mL AdCTLA on day 1 after corneal transplantation, serum CTLA4-Ig was measured by ELISA. In three of the rats tested, CTLA4-Ig diminished to undetectable levels before the onset of rejection. It is of interest that in one of the two other graft recipients, CTLA4-Ig levels remained high at day 45, despite allograft rejection at day 39, indicating that rejection can occur in the presence of CTLA4-Ig levels sufficiently high to block CD28/B7 costimulation (Fig. 7A). Serum containing CTLA4-Ig from a rat administered intravenous AdCTLA significantly decreased the T-cell proliferation in an MLR to less than 50% of that in control animals at 125 ng/mL CTLA4-Ig and to 10% at 500 ng/mL (Fig. 7B). This suggests that CTLA4-Ig circulating in the peripheral blood of graft recipients after intravenous AdCTLA administration is active and capable of prolonging allograft survival. The variability in serum CTLA4-Ig levels may reflect a difference in the immunogenic response of the host to the virus gene products, with prolonged expression being due to inhibition of antiadenovirus immune responses by CTLA4-Ig itself, as has been reported in a previous study.[26]

DISCUSSION

In this study we compared administration of CTLA4-Ig as a protein and as cDNA, using a recombinant viral vector. We compared the effect of both regimens on corneal graft survival after topical and intraperitoneal delivery. We first examined the expression of CD80 and CD86 costimulatory molecules after corneal transplantation. RT-PCR analysis showed increased expression of CD80 mRNA within the recipient and donor cornea immediately after transplantation. This probably reflects an influx of $CD80^+$ APCs in response to surgical trauma, because it was seen in both syngeneic and allogeneic grafts. CD86 expression was detected constitutively within the cornea, presumably in intraepithelial Langerhans cells. Differences in timing of expression of CD80 and CD86 may play an important role in allograft rejection.[27] On the one hand, the constitutive expression of CD86 suggests that it participates in initiating an immune response, thus playing an important role in determining whether a T cell contributes to an immune response or becomes anergic. On the other hand, CD80, which is expressed later, may serve to amplify or regulate the immune response.[28] Although we cannot exclude some changes in CD80–CD86 expression within the draining lymph nodes after transplantation, the high constitutive expression of CD80–CD86 molecules made such alterations difficult to detect.

Although CD80 and CD86 mRNA were detectable in cornea 16 hours after transplantation, neither CD80 nor CD86 protein was detectable on day 1, and only a trace amount of CD86 was detectable on day 5 after transplantation. It is likely that this reflects both the low level of early expression of CD80 and CD86 in the cornea after transplantation and also that, even in lymphoid tissue, a highly sensitive immunohistochemical technique is required to detect expression.[29] Overall, both CD80 and CD86 mRNA and protein expression was greater in the

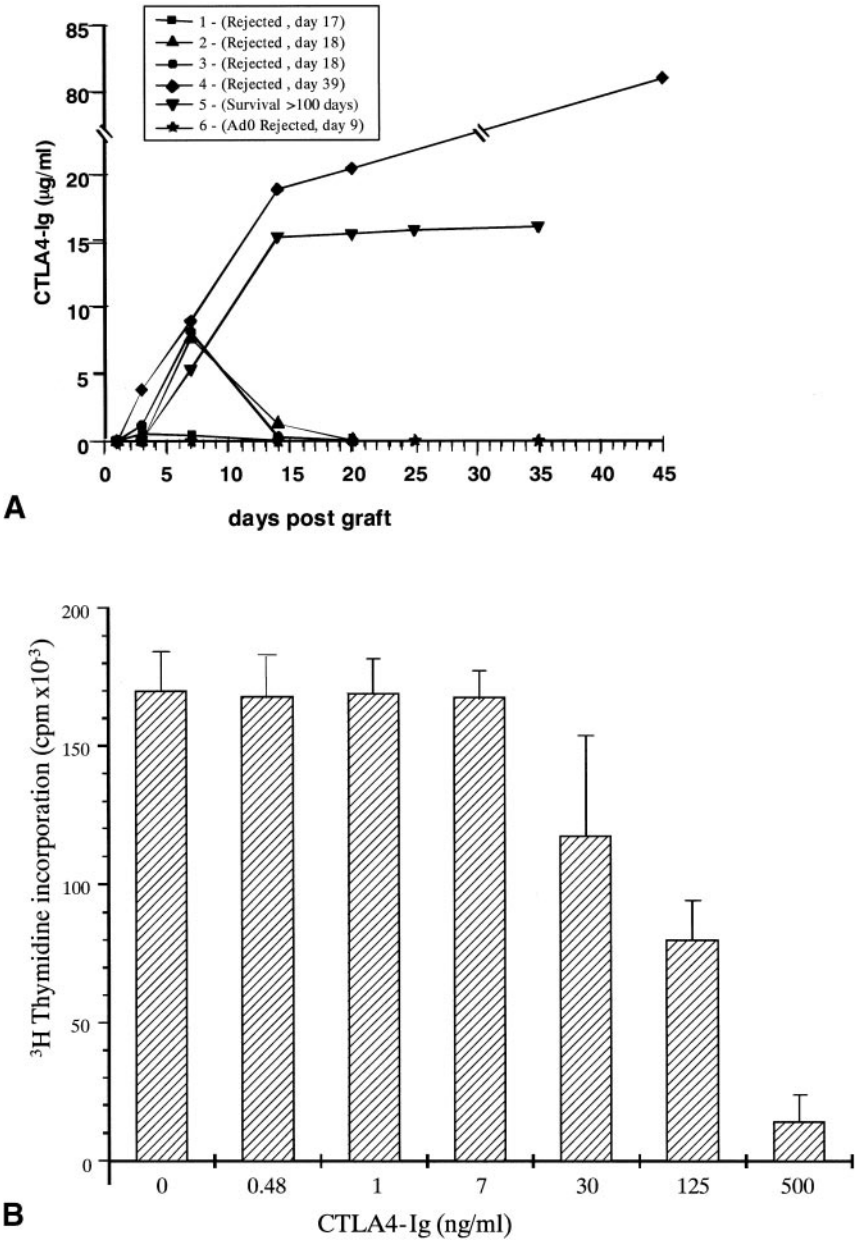


FIGURE 7. CTLA4-Ig in serum after intravenous adenovirus-mediated gene transfer. (**A**) AdCTLA 1×10^9 PFU/mL was injected on day 1 after corneal transplantation. Serum samples were collected at the indicated times and CTLA4-Ig quantified by ELISA. Concentrations declined to below the level of detection at the onset of rejection (days 17, 18, and 18, respectively) in rats 1, 2 and 3. In rats 4 and 5, levels remained high, and both had significantly longer allograft survival (39 and more than 100 days, respectively). CTLA4-Ig was undetectable after Ad0 injection. *Symbols* are shown with the subject number of each rat. (**B**) Biological activity of adenovirus-derived CTLA4-Ig in serum was determined by MLR. Diluted serum from a rat treated with 1×10^9 PFU/mL intravenous AdCTLA after transplantation was added to a BN-LEW MLR. [^{3}H]Thymidine incorporation of responder cells on day 3 after incubation was significantly decreased in a dose-dependent manner, compared with control subjects. This indicates that CTLA4-Ig detected by ELISA in the peripheral blood of graft recipients after intravenous AdCTLA administration was biologically active. Results are expressed as the mean ± SD of triplicates.

cervical draining lymph nodes than in the cornea after transplantation. This finding suggests that after transplantation T-lymphocytes are more likely to become activated through CD28 signaling in the draining lymph nodes than in the cornea itself.

One of the most striking findings is that corneal allograft survival was only moderately prolonged after systemic treatment with CTLA4-Ig. This has been observed in other animal models of corneal transplantation.[14,15] We used the same BN–LEW strain combination and treatment regimen that has been shown to markedly prolong cardiac allograft survival.[25] The result suggests that use of systemic treatment with CTLA4-Ig to block costimulation through CD28 is of less benefit in transplantation of cornea than of solid organs. There are several unique features of corneal transplantation that may be relevant to this. The first is that corneal allograft rejection is dominated by the indirect pathway of allorecognition,[30,31] because donor cornea is relatively devoid of dendritic cells, which express CD80 and CD86 molecules. However, as even in cardiac transplantation the indirect pathway is a component of allorecognition, and in addition there is no reason to suppose that CTLA4-Ig would not have an effect on the costimulatory molecules of the infiltrating recipient APCs that initiate the indirect pathway, it seems unlikely that this is the explanation. It may be that the reformation of the blood–aqueous barrier soon after transplantation excludes protein from the graft, thus reducing its effectiveness. However, this was not the case when the CTLA4-Ig was administered as an adenoviral construct.

Graft survival was prolonged when CTLA4-Ig was administered topically. This suggests that anatomic considerations may be important. However, the maximal benefit was seen with an intermediate dose of the fusion protein (2 μg/mL), with higher and lower concentrations being less effective. A reason for the lesser effect on graft survival at the higher concentration of CTLA4-Ig may be inhibition of CD80–CD86's engagement with the negative regulator CTLA-4 at this dose.

Other groups have failed to see any benefit, either of topical CTLA4-Ig administration in mice (after subconjunctival injection)[14] or of incubation of rabbit corneas in the fusion protein before transplantation.[15] The latter investigators, however,

saw prolonged survival if the corneas were transplanted into a vascularized recipient corneal bed, which confers a higher risk of rejection. The absence of effect on graft survival may be related to the large size of the CTLA4-Ig molecule (100–200 kDa) hindering penetrance through the tight junctions of the corneal epithelium.[32,33] In our experiments, we removed the epithelium, which may enhance, to some extent, diffusion of protein into the corneal stroma.

A single intravenous administration of adenovirus encoding CTLA4-Ig had a much more pronounced effect on allograft survival than topical administration, with all allografts surviving longer than null vector control grafts and one graft surviving more than 100 days. Expression of the virus-derived protein is most likely to occur in the liver.[34] As reported by Guillot et al.,[35] we detected high levels of active protein in the serum of animals. It is interesting that in some recipients, rejection was seen even in the face of circulating CTLA4-Ig, at high enough levels to block an MLR. This suggests that either the protein is unable to reach the appropriate anatomic site or that there is a CD28-independent costimulatory pathway that operates during corneal allograft rejection. Notwithstanding, we assume that the improvement in survival after cDNA administration, when compared with protein administration, is as a result of sustained high-level production of the molecule.

Topical administration of the cDNA had a lesser effect on graft survival. Levels of fusion protein produced in this way are lower and may not be high enough to be effective. In addition, insufficient protein may reach draining lymph nodes, where costimulatory interaction of APCs and T cells would be expected to take place. Finally, there may be toxic or proinflammatory effects of virus infection that are deleterious to graft survival. Differential survival of Ad0-infected corneas would to some extent control for such virus-specific effects, but we have reported this finding in a rabbit model of corneal grafting.[36] However, in experiments not described herein, we exposed rat corneas to the same concentrations of adenovirus used in the reported experiments, and no toxic effect on the endothelium was found, although higher concentrations (1×10^9 PFU/mL) showed marked cytopathogenicity. The use of less immunogenic, possibly nonviral, vectors may be necessary if topical pretransplantation modification of the donor cornea is to be effective.

In summary, we have demonstrated the capacity of the CTLA4-Ig fusion protein to prolong corneal allograft survival by treatment of either the recipient in vivo or the donor tissue ex vivo before grafting. Although the effect of systemic treatment with intraperitoneally administered protein on graft survival was less than that reported in vascularized organ allografts, corneal graft survival was significantly prolonged after a single administration of viral vector expressing CTLA4-Ig. This gene-based therapeutic strategy is highly attractive, because it demonstrates the persistence of gene expression without the need for repeated administration. Further studies on genes encoding CTLA4-Ig in less immunogenic vectors[37] and possibly in combination with other costimulation-blocking constructs hold considerable promise.

References

1. Price FW Jr, Whitson WE, Collins KS, Marks RG. Five year corneal graft survival: a large, single-center patient cohort. *Arch Ophthalmol.* 1993;111:799–805.
2. Williams KA, Muehlberg SM, Lewis RF, Coster DJ. How successful is corneal transplantation? A report from The Australian Corneal Graft Register. *Eye*. 1995;9:219–227.
3. Marrack P, Kappler J. The T cell receptor. *Science.* 1987;238:1073–1079.
4. Linsley PS, Brady W, Grosmaire L, Aruffo A, Damle NK, Ledbetter JA. Binding of the B cell activation antigen B7 to CD28 costimulates T cell proliferation and interleukin 2 mRNA accumulation. *J Exp Med.* 1991;173:721–730.
5. Walunas TL, Lenschow DJ, Bakker CY, et al. CTLA-4 can function as a negative regulator of T cell activation. *Immunity* 1994;1:405–413.
6. Krummel MF, Allison JP. CD28 and CTLA-4 deliver opposing signals which regulate the response of T cells to stimulation. *J Exp Med.* 1995;182:459–465.
7. Schwartz RH. A cell culture model for T lymphocyte clonal anergy. *Science.* 1990;248:1349–1356.
8. Wallace PM, Rodgers JN, Leytze GM, Johnson JS, Linsley PS. Induction and reversal of long-lived specific unresponsiveness to a T-dependent antigen following CTLA4Ig treatment. *J Immunol.* 1995;154:5885–5895.
9. Lin H, Bolling SF, Linsley PS, et al. Long-term acceptance of major histocompatibility complex mismatched cardiac allografts induced by CTLA-4Ig plus donor-specific transfusion. *J Exp Med.* 1993;178:1801–1806.
10. Sayegh MH, Akalin E, Hancock WW, et al. CD28–B7 blockade after alloantigenic challenge in vivo inhibits Th1 cytokines but spares Th2. *J Exp Med.* 1995;181:1869–1874.
11. Matsumura Y, Zuo XJ, Prehn J, et al. Soluble CTLA4Ig modifies parameters of acute inflammation in rat lung allograft rejection without altering lymphocytic infiltration or transcription of key cytokines. *Transplantation.* 1995;59:551–558.
12. Lenschow DJ, Zeng Y, Thistlewaite JR, et al. Long-term survival of xenogeneic pancreatic islet cell grafts induced by CTLA4Ig. *Science.* 1992;257:789–792.
13. Steurer W, Nickerson PW, Steele AW, Steiger J, Zheng XX, Strom TB. Ex vivo coating of islet cell allografts with murine CTLA4/Fc promotes graft tolerance. *J Immunol.* 1995;155:1165–1174.
14. Hoffman F, Zhang EP, Wachtlin J. Inhibition of corneal allograft reaction by CTLA4-Ig. *Graefes Arch Clin Exp Ophthalmol.* 1997;235:535–540.
15. Gebhardt BM, Hodkin M, Varnell ED, Kaufman HE. Protection of corneal allografts by CTLA-4 Ig. *Cornea.* 1999;18:314–320.
16. Williams KA, Coster DJ. Penetrating corneal transplantation in the inbred rat: a new model. *Invest Ophthalmol Vis Sci.* 1985;26:23–30.
17. Larkin DFP, Calder VL, Lightman SL. Identification and characterisation of cells infiltrating the graft and aqueous humour in rat corneal allograft rejection. *Clin Exp Immunol.* 1997;107:381–392.
18. King WJ, Comer RM, Hudde T, Larkin DFP, George AJT. Cytokine and chemokine expression kinetics after corneal transplantation. *Transplantation.* 2000;70:1225–1233.
19. Meagher L, Mahiouz D, Sugars K, et al. Measurement of mRNA for E-selectin, VCAM-1 and ICAM-1 by reverse transcription and the polymerase chain reaction. *J Immunol Methods.* 1994;175:237–246.
20. Bouaboula M, Legoux P, Pessegue B, et al. Standardization of mRNA titration using a polymerase chain reaction method involving co-amplification with a multispecific internal control. *J Biol Chem.* 1992;267:21830–21838.
21. Lane P, Gerhard W, Hubele S, Lanzavecchia A, McConnell F. Expression and functional properties of mouse B7/BB1 using a fusion protein between mouse CTLA4 and human gamma 1. *Immunology.* 1993;80:56–61.
22. Oral HB, Larkin DFP, Fehervari Z, et al. Ex vivo adenovirus-mediated gene transfer and immunoregulatory protein production in human cornea. *Gene Ther.* 1997;4:639–647.
23. Ramirez F, Silva A. Glucocorticoids enhance concanavalin A-induced mitogenic response through the inhibition of nitric oxide production. *Immunology.* 1997;90:66–73.
24. Larkin DFP, Oral HB, Ring CJA, Lemoine NR, George AJT. Adenovirus-mediated gene transfer to the corneal endothelium. *Transplantation.* 1996;61:363–370.
25. Turka LA, Linsley PS, Lin H, et al. T-cell activation by the CD28 ligand B7 is required for cardiac allograft rejection in vivo. *Proc Natl Acad Sci USA.* 1992;89:11102–11105.
26. Olthoff KM, Judge TA, Gelman AE, et al. Adenovirus-mediated gene transfer into cold-preserved liver allografts: survival pattern and unresponsiveness following transduction with CTLA4-Ig. *Nat Med.* 1998;4:194–200.

27. Freeman GJ, Boriello F, Hodes RJ, et al. Cloning of B7–2: ACTLA-4 counter-receptor that costimulates human T cell proliferation. *Science.* 1993;262:909–911.
28. Lenschow DJ, Walunas TL, Bluestone JA. CD28/B7 system of T cell costimulation. *Annu Rev Immunol.* 1996;14:233–258.
29. Damoiseaux JGMC, Yagita H, Okumura K, van Breda Vriesman PJC. Costimulatory molecules CD80 and CD86 in the rat; tissue distribution and expression by antigen-presenting cells. *J Leukoc Biol.* 1998;64:803–809.
30. Williams KA, White MA, Ash JK, Coster DJ. Leukocytes in the graft bed associated with graft failure: analysis by immunohistology and actuarial graft survival. *Ophthalmology.* 1989;96:38–44.
31. Neiderkorn JY. Immune privilege and immune regulation in the eye. *Adv Immunol.* 1990;48:191–226.
32. Burstein NL, Anderson JA. Corneal penetration and ocular bioavailability of drugs. *J Ocul Pharmacol.* 1985;1:309–326.
33. Allansmith M, de Ramus A, Maurice D. The dynamics of IgG in the cornea. *Invest Ophthalmol Vis Sci.* 1979;18:947–955.
34. Kita Y, Li XK, Ohba M, et al. Prolonged cardiac allograft survival in rats systemically injected with adenoviral vectors containing CTLA4-Ig gene. *Transplantation.* 1999;68:758–766.
35. Guillot C, Mathieu P, Coathalem H, et al. Tolerance to cardiac allografts via local and systemic mechanisms after adenovirus-mediated CTLA4Ig expression. *J Immunol.* 2000;164:5258–5268.
36. Rayner SA, Larkin DFP, George AJT. TNF receptor secretion after ex vivo adenoviral gene transfer to cornea and effect on in vivo graft survival. *Invest Ophthalmol Vis Sci.* 2001;42:1568–1573.
37. Hudde T, Rayner SA, Comer RM et al. Activated polyamidoamine dendrimers, a non-viral vector for gene transfer to the corneal endothelium. *Gene Ther.* 1999;6:939–943.