Modulation of Costimulation by CD28 and CD154 Alters the Kinetics and Cellular Characteristics of Corneal Allograft Rejection

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Purpose. To examine the effect of modulating the lymphocyte costimulation pathways through CD28 and CD154 (CD40 ligand) in a model of corneal allograft rejection, with particular interest in changes in the observed features of rejection.

Methods. CD28 knock-out (CD28KO) and wild-type BALB/c control mice received corneal grafts from fully major histocompatibility complex (MHC)–mismatched C3H donors and were treated with CTLA4-Ig and/or anti-CD154 Ab on days 0, 2, and 4 after transplantation. Proliferation of BALB/c and CD28KO T cells in response to C3H stimulators was examined in a mixed lymphocyte reaction (MLR) in the presence of CTLA4-Ig or anti-CD154 Ab.

Results. Corneal allograft survival in wild-type BALB/c mice (median survival time [MST] 14 days) was significantly prolonged by blockade of the costimulatory pathways with CTLA4-Ig or anti-CD154 Ab (MST 21 days and 25 days respectively). MST in recipients treated with CTLA4-Ig and anti-CD154 Ab in combination was 29 days, not significantly longer than graft survival in single-treatment groups. MST in CD28KO recipients was 46 days and was not prolonged after treatment with anti-CD154 Ab (MST, 43 days). A similar result was found in the MLR, in which anti-CD154 Ab had no effect on proliferation of CD28KO compared with wild-type T cells. In CTLA4-Ig–treated CD28KO, grafts were rejected at an accelerated tempo, similar to that in wild-type BALB/c recipients (MST 16 days). More severe graft injury after the onset of rejection in untreated allograft recipients was accompanied by a higher number of graft-infiltrating $CD45^+$ cells, but similar proportions of $CD4^+$ and $CD8^+$ cells.

Conclusions. CD28- and CD154-mediated costimulation have significant functional roles in corneal allograft rejection. Agents that modulate CD28 and CD154 pathways delay onset and reduce the severity of observed allograft rejection. However, their use in combination did not have an additive effect, MLR data indicating that the CD40–CD154 system depends on a functioning CD28 costimulatory pathway. (*Invest Ophthalmol Vis Sci.* 2003;44:3899–3905) DOI:10.1167/iovs.03-0084

Corneal transplantation is the only treatment for many blinding disorders, and the cornea is the most commonly transplanted tissue. However, despite a comparative degree of immune privilege, allogeneic rejection is the commonest cause of corneal graft failure. As in other transplanted tissues, corneal allograft rejection is primarily T-cell mediated.[1,2] Current strategies to prevent rejection in those recipients perceived to be at high risk rely on systemically administered immunosuppressive agents, the adverse effects of which are not justified in many patients in whom disease is unilateral.[3,4] Because for this reason so many patients are denied the opportunity for transplantation, more specific immunomodulatory treatment would allow such patients to receive a corneal transplant.

Allograft rejection is primarily dependent on activation of alloreactive T lymphocytes. After interaction of antigen-major histocompatibility complex (MHC) with the T-cell receptor–CD3 complex, which gives specificity to the immune response, costimulation through one or more additional receptor-ligand pairs has been shown to be necessary for full activation of naive T cells.[5] The interaction of CD28 with its antigen-presenting cell (APC) ligands CD80/CD86 is one of the dominant costimulatory pathways, blockade of which has been demonstrated in many studies to result in prevention of allograft rejection and other immune responses.[6–9] Although survival is prolonged in allograft models in many species, induction of tolerance has been more difficult to demonstrate, suggesting that costimulation can be effected by one of the CD28-independent pathways now recognized. In this respect, interaction of CD154 (CD40L) with the CD40 receptor on APCs is a likely candidate. On activation, T cells express CD154, and the CD40 receptor has been shown to upregulate several costimulatory activities of APC of importance in a range of T-cell–dependent immune responses. This pathway also plays a critical role in allograft rejection, as inhibition with an anti-CD154 antibody significantly prolongs murine cardiac,[10] and primate islet[11] allograft survival. Larsen et al.[12] reported long-term acceptance of both of cardiac and skin grafts, indicating additive effects of blockade of both the CD28 and CD154 costimulatory pathways.

Selective blockade of costimulation through either CD28 or CD154 has been shown to prolong corneal allograft survival.[13,14] In the study of Qian et al.,[14] anti-CD154 antibody was administered weekly for 8 weeks, and at the termination of the treatment period, graft survival was examined. We examined the effect of blockade of the CD28 and CD154 pathways individually and in combination in murine corneal transplantation. Because loss of donor corneal transparency is the end point in corneal allograft rejection and can be graded by direct observation, comparison could readily be made between rapidity of rejection onset and severity of rejection parameters in different recipient treatment groups. This is not possible in

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other transplantation models, in which the interval to graft rejection is the sole end point. This model could therefore be used to examine the effects of costimulatory blockade on phenotypic correlates of rejection.

Methods

Generation of CTLA4-Ig

The CTLA4-Ig fusion protein was produced with Chinese hamster ovary cells transfected with cDNA encoding human CTLA4 and human IgG Fc. Cells were cultured in RPMI (Invitrogen/Gibco, Paisley, UK), 1% IgG-depleted FCS (Globepharm, Esher, UK), 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 5 mM glucose and selected in the presence of 1 μg/mL gentamicin (all Invitrogen/Gibco). The protein was purified by protein G chromatography (Sigma-Aldrich, Poole, UK).

Generation of Anti-CD154 Antibody

A B-cell hybridoma producing hamster mAb against mouse CD154 (MR1, IgG_{2a}) was obtained from American Type Culture Collection (ATCC, Manassas, VA). Hybridoma cells were cultured in RPMI 1640, 1% IgG-depleted FCS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. Antibody was purified by protein G chromatography (Sigma-Aldrich).

ELISA for CTLA4-Ig and Anti-CD154 Ab Concentration

A 96-well microtiter plate (Nalge Nunc International, Taastrup, Denmark) was coated with 2.5 μg/mL rabbit anti-human IgG (Fc-specific; Dako, Glostrup, Denmark) or 2.5 μg/mL mouse anti-hamster IgG (BD Pharmingen, Cowley, UK) capture Ab in 0.1 M Na_2HPO_4 (pH 9.6). After overnight incubation at 4°C, the plate was washed with PBS and 0.1% Tween and blocked with 2% milk for 45 minutes at 37°C. Plates were washed again, and the standards (human IgG; Zymed, Cambridge, UK; hamster IgG, Serotec, Kidlington, UK) or samples were added in 100-μL volumes to each well in triplicate, incubated for 2 hours at room temperature, and washed again. The secondary Ab, biotin-conjugated goat anti-human IgG (1:2000; Sigma-Aldrich) or biotin-conjugated mouse anti-hamster IgG (2 μg/mL; BD Pharmingen) was then added and incubated for 1 hour at room temperature. In the case of CTLA4-Ig, the capture Ab was preincubated with 5% rabbit and 5% goat serum (Sigma-Aldrich) in PBS and 0.1% Tween for 30 minutes at room temperature to block a nonspecific cross-reaction. After four washes, 100 μL of streptavidin-horseradish peroxidase (HRP) 1:10,000 (Dako) in PBS and 0.1% Tween was added to each well for 15 minutes at room temperature. After four additional washes, 50 μL of TMB substrate (Zymed) was added to the wells and incubated for 5 minutes at room temperature. The reaction was stopped by the addition of 50 μL of 1 M H_2SO_4. Plates were read at 450 nm with a microplate reader. The ELISA detection limit was 4 ng/mL for CTLA4-Ig and 1 ng/mL for anti-CD154 Ab.

Animals

Inbred adult female adult BALB/c mice ($H\text{-}2^d$; Harlan Olac, Bicester, UK) and female BALB/c CD28-deficient ($H\text{-}2^d$) mice,[15] obtained from The Jackson Laboratory (Bar Harbor, ME) were used as graft recipients. Targeted deletion was confirmed by PCR analysis for the CD28 sequence in wild-type BALB/c and knock-out (KO) mice. Female C3H/He ($H\text{-}2^k$; Harlan Olac) mice were used as allogeneic and BALB/c as syngeneic donors. Animals were maintained in a specific pathogen-free facility and treated in accordance to the UK regulations for care of experimental animals. Animal studies were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Mixed Lymphocyte Reaction

The MLR was performed as previously described,[16] with some modification. In brief, the spleen and major lymph nodes (cervical, inguinal, and abdominal) of BALB/c CD28KO mice and C3H mice were used to collect APCs and T cells, which were separated with a nylon wool column. For purification, T cells were resuspended with 1 mL M5/114 supernatant (anti-I-A^d antibody) and 1 mL ADH4 supernatant (anti-CD8 antibody) for 30 minutes on ice. The cells were then washed twice (635*g* for 5 minutes) and incubated with goat anti-rat magnetic beads (Dynabeads; Dynal, Bromborough, UK) twice for 30 minutes a on rotator at 4°C. Stimulator cells (APCs) were irradiated with 30 Gy. The stimulator (C3H) and responder ($CD4^+$ cells from BALB/c mice) cells were resuspended at 2×10^5 cells at a 1:1 ratio. The plate was cultured at 37°C in 5% CO_2 for 72 hours before being pulsed with 1 μCi/well [^{3}H]-TdR for 16 hours.

Orthotopic Corneal Transplantation and Definition of Corneal Graft Rejection

Orthotopic corneal transplantation was performed in the right eye of all animals (6–10 weeks), as described by Zhang et al.,[17] with minor modifications. A 2.5-mm diameter donor corneal graft was sutured into a 2.0-mm recipient corneal bed with one continuous 11-0 nylon suture (Ethicon, Somerville, NJ). At the completion of the procedure, the anterior chamber was reformed with sterile saline and chloramphenicol ointment (Martindale Pharmaceuticals, Brentwood, UK) applied on the cornea. The eyelids were sutured closed to protect the cornea for the initial 48 hours. At that time and thrice weekly thereafter, the eye was examined by operating microscope. Technically satisfactory grafts had corneal opacity grade 0 to 1 at all times until suture removal on day 7. All other graft recipients, and those with intraocular hemorrhage or cataract were excluded.

Corneal transparency is an indicator of corneal endothelial function and of graft endothelial injury. The onset of graft rejection was diagnosed when corneal opacity increased to grade 3 (no iris vessels visible) in a graft previously clear after transplantation (Fig. 1). Grading of corneal opacity was as described by Sonoda and Streilein[18] with minor modifications, as follows: 0, completely transparent cornea; 1, minimal corneal opacity, but iris vessels easily visible; 2, moderate corneal opacity, iris vessels still visible; 3, moderate corneal opacity, only pupil margin visible; 4, complete corneal opacity, pupil not visible.

In Vivo Treatment of Graft Recipients

The following were administered to recipient animals by intraperitoneal injection of 200 μg on days 0, 2, and 4 after transplantation: huCTLA4-Ig, anti-CD154 antibody, and human IgG or hamster IgG as negative controls for CTLA4-Ig and anti-CD154, respectively.

Immunohistology

Rejection was confirmed by end point histology in all graft recipients. Corneas were excised on the day of observed onset of rejection. The tissue was snap frozen in liquid nitrogen and stored at −70°C or fixed in 10% formalin and embedded in paraffin wax before staining with hematoxylin and eosin (H&E) or immunohistochemistry as follows. Paraffin sections were incubated with 10 μg/mL rat anti-mouse CD45 mAb; cryostat sections were incubated with 1 μg/mL rat anti-mouse CD4 or 3 μg/mL rat anti-mouse CD8 monoclonal antibody (both from BD Pharmingen). Sections were then washed, incubated with biotinylated rabbit anti-rat IgG secondary Ab (2 μg/mL, Vector Laboratories, Orton Southgate, UK), washed, and incubated with avidin-biotin complex (ABC; Vector Laboratories). The chromogen 3,3′-diaminobenzidine (DAB; Dako) was then applied before hematoxylin counterstaining. Positive controls were sections of mouse spleen; negative controls were sections incubated with rat IgG_{2a} or IgG_{2b} (R&D Systems, Abingdon, UK) as the primary Ab.

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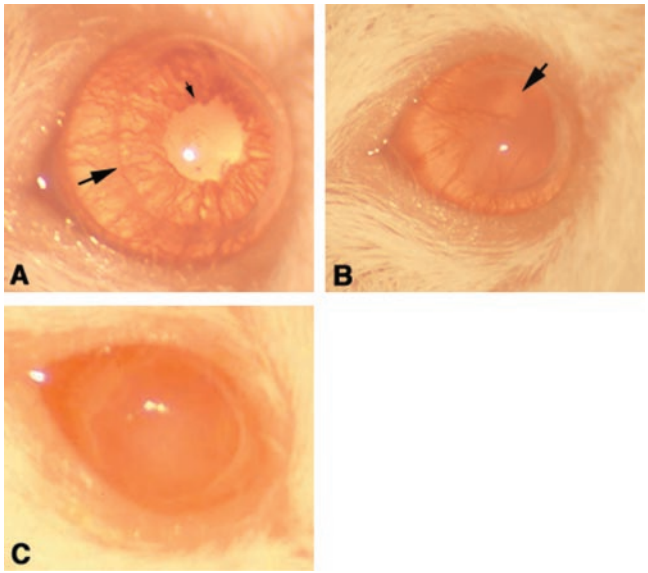

FIGURE 1. Grading of graft transparency. Grading of the corneal allograft was according to transparency. Grade 0 is a fully transparent graft, through which the iris vessels and pupil margin (*small arrow*) are easily visible. *Large arrow*: graft margin (**A**). Rejection was diagnosed at grades 3 (**B**; only the pupil margin visible; *arrow*) and 4 (**C**; complete corneal opacity).

Scoring of Positive Immunostaining in the Tissue

Positive-stained cells were counted in three adjacent light microscopic fields in the center of the corneal graft, using 100× objective magnification. Counts were made in three nonserial sections of each specimen in three corneas from each treatment group. Cryostat sections were evaluated semiquantitatively as follows: 0, no staining; 1, 10% or less of cells stained; 2, 11% to 30% stained; 3, 31% to 50% stained; 4, 51% to 80% stained; 5 more than 80% stained.

Statistical Analysis

Actuarial graft survival was analyzed using the Kaplan-Meier survival method.[19] The log-rank test was used to examine for statistical differences between the groups. Student's *t*-test was used to analyze immunohistochemistry data and corneal opacity time. $P < 0.05$ was defined as statistically significant and only significant probabilities are shown.

RESULTS

Corneal Allograft Survival

Syngeneic grafts in wild-type BALB/c recipients ($n = 8$) remained transparent throughout the 100-day observation period. Survival of C3H donor grafts in unmodified BALB/c recipients ranged from 12 to 36 days, and MST was 14 days ($n = 16$).

CD28 Pathway Modulation. MST of allografts in CTLA4-Ig–treated BALB/c mice ($n = 8$) was 21 days, significantly prolonged compared with untreated ($P < 0.003$) and human IgG–treated recipients ($n = 6$, MST 12 days). MST in untreated CD28KO mice ($n = 6$) was 46 days, significantly longer than in wild-type mice ($P < 0.001$). In contrast, allograft survival in CTLA4-Ig–treated CD28KO mice ($n = 6$) was shortened (MST 16 days), consistent with blockade by CTLA4-Ig of negative signaling through CD152 (Fig. 2A).

CD40 Pathway Modulation. Allograft survival in BALB/c recipients treated with anti-CD154 Ab ($n = 8$) and hamster IgG ($n = 6$) was a median of 25 ($P < 0.008$ compared with untreated BALB/c recipients) and 13 days, respectively (Fig. 2B). This indicates a significant effect of signaling through CD40.

CD28+CD40 Pathway Modulation. Combined treatment with CTLA4-Ig and anti-CD154 antibody ($n = 7$, MST 29 days) significantly extended allograft survival compared with untreated BALB/c recipients ($P < 0.0035$), but not compared with treatment with each agent alone. Longer allograft survival was found in CD28KO mice treated with anti-CD154 ($n = 6$, MST 43 days) compared with untreated wild-type BALB/c mice ($P < 0.001$), but not compared with untreated CD28KO mice (Fig. 2C).

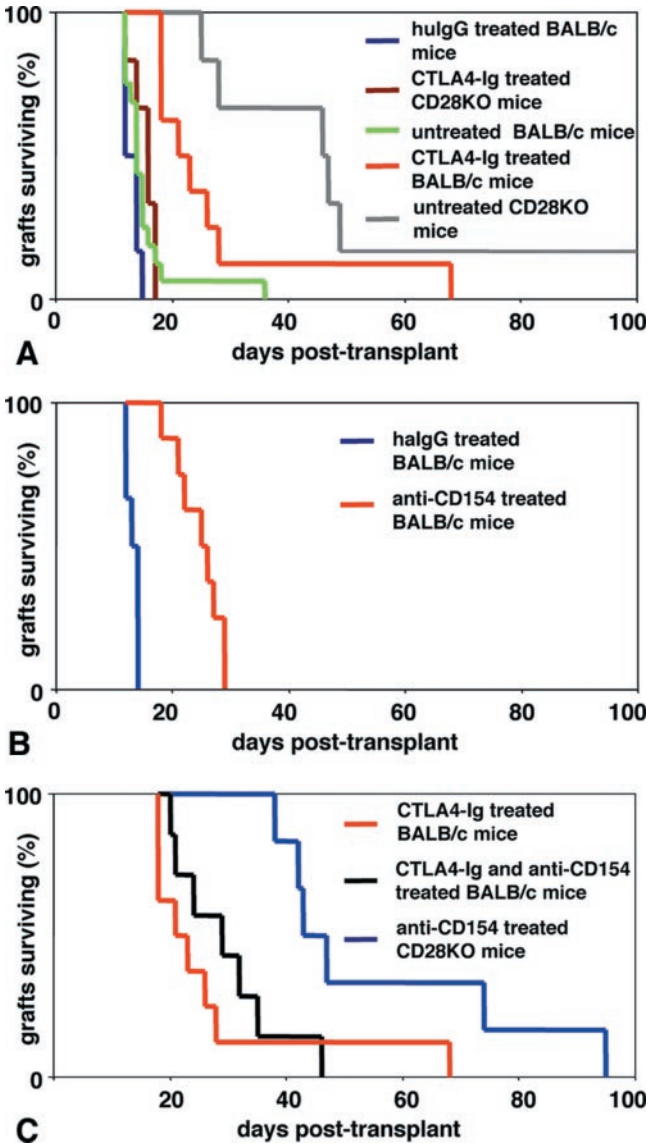

FIGURE 2. Extension of actuarial corneal allograft survival by administration of 200 μg CTLA4-Ig and/or 200 μg anti-CD154 on days 0, 2, and 4 after transplantation. (**A**) Modulation of the CD28-CD80/86 pathway. MST in untreated BALB/c recipients was 14 days. Allograft survival in CTLA4-Ig–treated (MST 21 days) and CD28KO (MST 46 days) recipients was significantly prolonged. Survival in CTLA4-Ig–treated CD28KO was similar to that in untreated BALB/c recipients (MST 16 days). MST in human IgG–treated recipients was 12 days. (**B**) Modulation of the CD40–CD154 pathway. MST in anti-CD154 treated mice was 25 days. Hamster IgG had no effect on graft survival (MST 13 days). (**C**) Combination CTLA4-Ig and anti-CD154 did not result in significantly longer survival (MST 29 days) than CTLA4-Ig alone. Anti-CD154 did not prolong survival in CD28KO recipients (MST 43 days).

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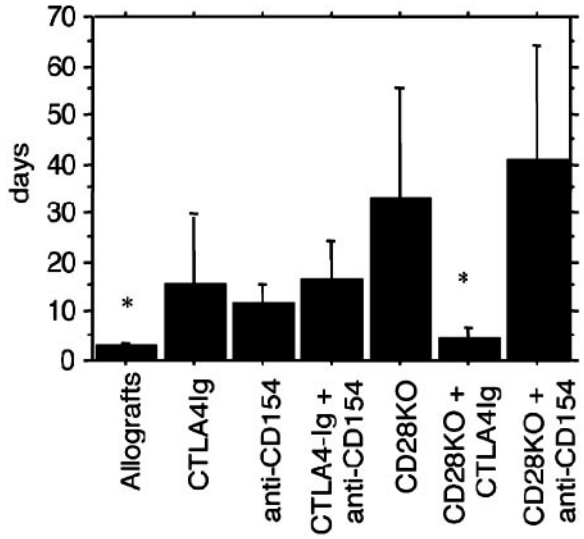

FIGURE 3. Progression from early to advanced corneal opacity. The interval from initial detection of corneal opacity (grades 1, 2) until rejection (grades 3, 4) was recorded. This was significantly shorter (*) in untreated allograft recipients and CTLA4-Ig–treated CD28KO recipients.

Duration of Corneal Graft Opacity before Diagnosis of Rejection

The interval from initial detection of corneal opacity (grades 1, 2), until diagnosis of rejection (grades 3 or 4) varied between groups. Whereas in untreated allograft recipients this interval was 2.7 ± 0.3 days, it was significantly prolonged to 15.6 ± 5.8 days in animals with CTLA4-Ig treatment ($P < 0.05$), 11.9 ± 1.5 days with anti-CD154 Ab ($P < 0.001$), and 16.6 ± 3.1 days with CTLA4-Ig and anti-CD154 Ab ($P < 0.006$). In untreated CD28KO mice, this interval was 32.8 ± 8.8 days and in CD28KO mice with anti-CD154 antibody treatment, 41 ± 8.9 days. CTLA4-Ig–treated CD28KO mice had a corneal opacity time before diagnosis of rejection similar to those with untreated allografts (4.2 ± 0.9 days; $P < 0.31$ vs. allografts; Fig. 3).

Grade of Corneal Opacity at Diagnosis of Graft Rejection

Because on a graft opacity scale of 0 to 4, the minimum score for rejection diagnosis was 3 and grafts were examined on alternate days, a grade of 4 rather than 3 on the day of rejection diagnosis indicates more rapid onset and severe graft injury. Modulation of costimulation resulted in less severe allograft injury, as determined by lower grades of corneal opacity in CD28KO recipients treated with CTLA4-Ig or anti-CD154 than was apparent after rejection onset in untreated BALB/c mice. In untreated recipients, 9 (57%) of 16 corneal allografts had a grade-4 opacity on the day rejection onset was observed. Grade-4 opacity was found in one (12%) of eight CTLA4-Ig–treated BALB/c recipients, in two (25%) of eight anti-CD15–treated recipients, and in two (28%) of seven recipients treated with both agents. At the onset of rejection, allograft opacity scores in untreated CD28KO and anti-CD154–treated CD28KO recipients were grade 3 in all cases (100%). Scores in CTLA4-Ig treated CD28KO mice were grade 4 in two (33%) of six cases.

Mixed Lymphocyte Reaction

As expected, addition of CTLA4-Ig or anti-CD154 antibody showed a dose-dependent inhibition of proliferation of wild-type BALB/c but not CD28KO T cells. Inhibition was observed at concentrations ranging from 1 to 20 μg/mL CTLA4-Ig; equivalent concentrations of anti-CD154 antibody had less effect on proliferation (Fig. 4A, 4B). However, addition of CTLA4-Ig or anti-CD154 Ab did not alter the proliferation profile of CD28KO T-cells (Fig. 4C). Human and hamster IgG had negligible effects.

Histology and Immunohistochemical Staining

Rejected corneal allografts in untreated or IgG control-treated BALB/c mice showed heavy infiltration of $CD45^+$ inflammatory cells in the graft and the anterior chamber. In contrast, rejected grafts in CTLA4-Ig– and/or anti-CD154–treated recipients had a more sparse graft cellular infiltrate, and the anterior chamber was almost free of inflammatory cells. Rejected allografts in untreated CD28KO or anti-CD154–treated CD28KO mice also showed a reduced cell infiltrate in the graft and anterior chamber. In keeping with accelerated rejection in CTLA4-Ig–treated CD28KO graft recipients, graft inflammatory infiltrates were similar in all corneas to rejected allografts in BALB/c mice (Figs. 5, 6).

Semiquantitative analysis of CD4 and CD8 staining in the corneal allografts showed a reduced number of positive cells in the treated BALB/c and anti-CD154–treated CD28KO mice, compared with untreated and CTLA4-Ig–treated CD28KO allograft recipients. The proportions of $CD4^+$ and $CD8^+$ cells were unchanged (data not shown).

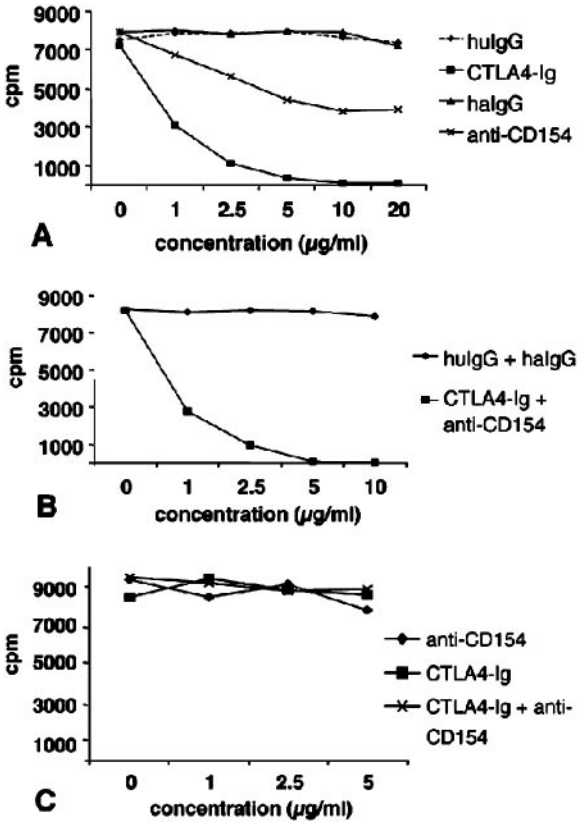

FIGURE 4. Effect of costimulatory blockade on MLR. CTLA4-Ig and/or anti-CD154 antibody concentration-dependent inhibition of proliferation in C3H-BALB/c MLR. Proliferation was reduced to negligible levels by CTLA4-Ig concentrations as low as 5 μg/mL. Less effect on proliferation was found at equivalent doses of anti-CD154 mAb (**A**). A combination of CTLA4-Ig and anti-CD154 had an effect on proliferation similar to that of CTLA4-Ig alone. Both human (hu) and hamster (ha) Ig had no effect (**B**). When the MLR was repeated with T cells from CD28KO mice (**C**), no inhibition occurred after addition of CTLA4-Ig or anti-CD154 mAb.

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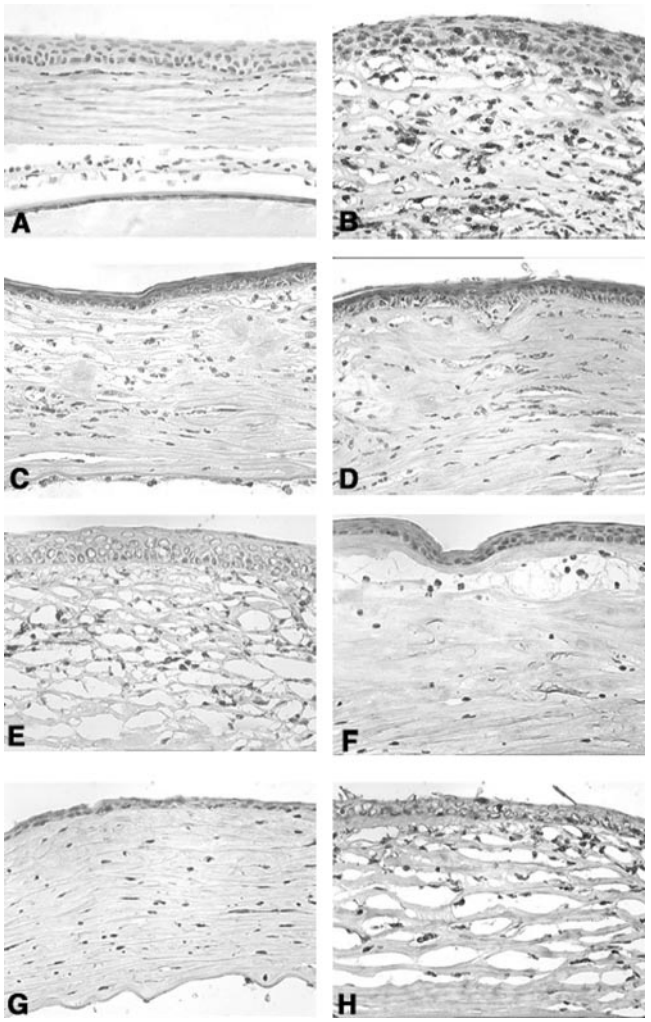

FIGURE 5. Graft-infiltrating $CD45^+$ cells in representative corneal transplant sections. (**A**) Syngeneic graft; (**B**) allograft in untreated BALB/c recipient; (**C**) allograft in CTLA4-Ig treated BALB/c; (**D**) allograft in anti-CD154–treated BALB/c; (**E**) allograft in CTLA4-Ig+anti-CD154 treated BALB/c; (**F**) allograft in untreated CD28KO recipient; (**G**) allograft in anti-CD154–treated CD28KO; (**H**) allograft in CTLA4-Ig–treated CD28KO.

DISCUSSION

Graft survival data reported herein indicate the functional importance of both the CD28–CD80/86 and CD154–CD40 costimulatory pathways in the allogeneic response to a cornea. However, blockade of each costimulatory pathway was found to result in a shorter prolongation of corneal graft survival than reported in other models, such as heart, skin, liver, or kidney.[12,20–22] In addition, we did not find an additive effect of combined blockade of CD28- and CD154-mediated costimulation.

We found a significant difference in allograft survival between CD28KO mice compared with wild-type BALB/c mice. Allograft survival in CD28KO recipients was longer than in CTLA4-Ig–treated mice, though only one cornea survived long-term. Variably extended survival of allografts of other tissues has been found in CD28KO mice: minimum prolongation of skin and longer survival of cardiac grafts. Long-term graft acceptance has not been reported.[23–25] Corneal allograft rejection in CD28KO mice treated with CTLA4-Ig was accelerated compared with that in untreated CD28KO animals, in accordance with reported results in cardiac transplants.[23,26] This is probably the result of blocking negative signaling interactions between CD80/86 and CD152 (CTLA4) on activated T cells. Alternatively, because CD152 is also expressed on regulatory T cells,[27,28] it is possible that the molecule is interfering with regulatory pathways controlling T-cell activation. In either case, these data highlight that $CD28^-$ T cells are capable of being activated but that CD80/86 interactions with CD152 normally prevent this from happening. These T cells must be activated in a CD28-independent manner. It is of interest that in a rat model, we have found rejection of corneal allografts, even in the presence of blocking levels of CTLA4-Ig,[13] indicating that other costimulatory pathways are operational in this model.

One of the most striking findings was the absence of additive effects in agents blocking CD40- and CD28-mediated costimulation. Synergy between these two reagents has been observed in several transplantation models and induced autoimmunity models.[12,21,29] Of particular interest therefore was the in vitro correlate that, in contrast to the predicted effect on proliferation of BALB/c responders, CD154 blockade had no effect on proliferation of CD28KO responders to C3H stimulators in an MLR. Thus, at least with respect to proliferation, the CD40–CD154 system depends on a functioning CD28 costimulatory pathway, and cross-regulation of the B7h-inducible costimulator (ICOS) and CD40–CD154 signaling is one possible explanation. Because CD28 signaling is essential for optimal T-cell ICOS expression,[30,31] this may be a functional phenotype of CD28 deficiency. No ICOS signaling is available to induce CD154 expression,[32,33] with the result that anti-CD154 Ab has little effect on the MLR.

Another striking feature of this model is the shorter survival of corneal allografts after treatment with CTLA4-Ig and anti-CD154 Ab, compared with other organs in the same strain combination using the same treatment regimens. Strain variation in allograft MSTs after CD40–CD28 blockade has been demonstrated.[34] However, the shorter survival of corneas may be explained in part by the sensitivity of corneal tissue. Diagnosis of corneal graft rejection is by direct visualization of reduced transparency. Clinical diagnosis in this way allows early rejection to be detected, but also less extensive rejection in which a minor degree of transparency remains, equating in this report to graft clarity grade 3 rather than 4. Accordingly,

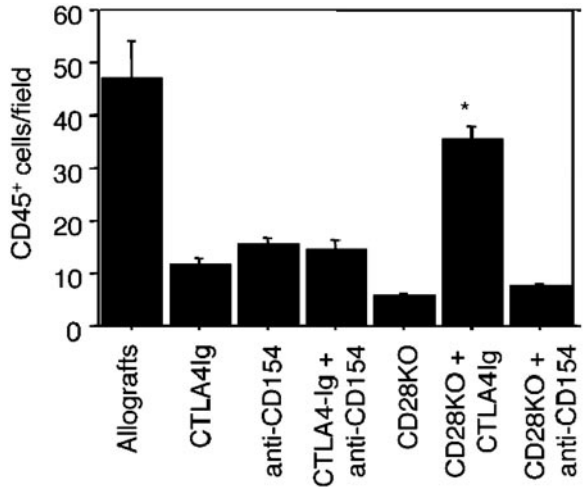

FIGURE 6. Graft-infiltrating $CD45^+$ cell counts. Positive cells in 100× magnification fields were counted and the mean ± SD from three corneas in each group is shown. Cell numbers were significantly lower in all treatment groups ($P < 0.01$) than in untreated allograft recipients, with the exception of CTLA4-Ig–treated CD28KO (*).

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the corneal model allows variations in the extent of allogeneic injury to be observed. We have shown that rejection led to submaximum loss of transparency in the CD28KO graft recipient groups treated with CTLA4-Ig and anti-CD154, but not untreated BALB/c. In contrast to cornea, the clinical diagnosis of rejection in heart transplantation is defined as cessation of heartbeat, and in skin transplantation as complete loss of visible graft epidermal tissue. Allogeneic graft injury insufficient to cause total loss of graft function in such models may not be categorized as rejection and account for some of the apparent greater survival of grafts in those models after costimulatory blockade.

The finding of altered clinical phenotype of rejection after costimulatory blockade reported herein was accompanied by reduction in the number of graft-infiltrating $CD45^+$ cells. At the time of rejection, there were significantly reduced numbers of $CD45^+$ cells in treated compared with untreated BALB/c mice. The number of $CD45^+$ cells was also reduced in allografts in CD28KO and anti-CD154 Ab–treated CD28KO. Further supporting the correlation of rejection phenotype with the number of graft-infiltrating $CD45^+$ cells, rejected grafts in CTLA4-Ig treated CD28KO mice contained a number of cells similar to that in rejected grafts in untreated BALB/c mice. The low number of $CD45^+$ cells may be due both to the blockade of costimulation resulting in failure of activation and the promotion of the apoptotic death of graft-infiltrating lymphocytes.[20]

Özkaynak et al.[35] have shown recently that blocking the CD40–CD154 pathway with an anti-CD154 mAb alone prolongs cardiac allograft survival, but does not prevent the development of transplant arteriosclerosis, interstitial fibrosis, or focal myocyte necrosis. These outcomes may be analogous to histologic features in rejected corneas. In those specimens from CTLA4-Ig– and/or anti-CD154–treated recipients in which a low number of $CD45^+$ cells was found, graft sections showed an increase in thickness and fibrosis with loss of keratocyte orientation, possibly equivalent to chronic graft rejection.[36] It is of interest that Özkaynak et al.[35] could prevent such fibrosis and transplant arteriosclerosis with the combined inhibition of the CD40–CD154 and ICOS pathways.

In recent years there has been considerable interest in using molecules to block interactions of CD40–CD154 and CD28–CD80/86, to prevent allograft rejection. Studies in primates have suggested that this approach has considerable promise for solid organ transplantation in humans. In most cases a short course of treatment is highly effective, although repeated doses enhance efficacy.[21] In this study we have shown that, although blockade of these pathways at the time of transplantation is effective in extending graft survival, it does not abolish allograft rejection. We suggest that this is in part because the fragile nature of the cornea allows the earliest effects of rejection to be readily observed. Corneal allografts therefore represent a highly sensitive model in which to assess the effectiveness of costimulatory blockade in preventing any degree of allogeneic rejection.

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