



# Blockade of CD40-CD154 Costimulatory Pathway Promotes Survival of Allogeneic Corneal Transplants

## Citation

Qian, Ying, Florence Boisgerault, Gilles Benichou and M.Reza Dana. 2001 "Blockade of CD40-CD154 Costimulatory Pathway Promotes Survival of Allogeneic Corneal Transplants". Investigative Ophthalmology & Visual Science 42, no.5: 987-94.

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# Blockade of CD40-CD154 Costimulatory Pathway Promotes Survival of Allogeneic Corneal Transplants

Ying Qian, Florence Boisgerault, Gilles Benichou, and M. Reza Dana

**PURPOSE.** To determine the effect of systemic anti-CD154 monoclonal antibody on the survival of orthotopic murine corneal transplants.

**METHODS.** BALB/c mice were used as recipients of syngeneic, multiple minor histocompatibility (H)-disparate, or major histocompatibility complex MHC-mismatched corneal transplants. Recipient beds were either avascular (normal risk) or neovascularized (high risk). Mice were randomized to receive either anti-CD154 antibody or control immunoglobulin by intraperitoneal injection at surgery and once weekly after surgery. After orthotopic corneal transplantation, all grafts were evaluated for signs of rejection by slit lamp biomicroscopy over 8 weeks. The high-risk transplants were continuously observed until week 18 after the therapy was discontinued at week 8. Allospecific delayed-type hypersensitivity (DTH) was evaluated after transplantation in high-risk graft recipients. Frequency of interferon (IFN)- $\gamma$ -secreting T cells in the hosts was measured by enzyme-linked immunospot (ELISPOT) assay.

**RESULTS.** In normal-risk transplantation, the 8-week survival rate improved from 25% in control mice to 88% in anti-CD154-treated hosts of minor H-disparate grafts ( $P = 0.0087$ ) and from 78% in control mice to 100% in anti-CD154-treated recipients of MHC-mismatched transplants ( $P = 0.177$ ). Of particular significance, in high-risk transplantation, anti-CD154 therapy dramatically enhanced the survival of both minor H- and MHC-disparate corneal transplants to 100% ( $P = 0.0001$ ) and 92% ( $P = 0.0002$ ), respectively. In addition, the anti-CD154-treated mice did not exhibit allospecific immunity. However, termination of anti-CD154 led to some loss in graft survival, especially among high-risk minor H-disparate grafts. The frequency of IFN- $\gamma$ -producing T cells was significantly reduced in anti-CD154-treated hosts.

**CONCLUSIONS.** Continuous suppression of the CD40-CD154 costimulatory pathway promotes the acceptance of corneal transplants, regardless of the degree of allospecificity or preoperative risk. The beneficial effect of anti-CD154 treatment may be due in part to inhibition of Th1-mediated responses. (*Invest Ophthalmol Vis Sci.* 2001;42:987-994)

The leading cause of corneal graft failure is T-cell-mediated immune rejection.<sup>1</sup> Current immunosuppressive drugs used to prevent or treat corneal graft rejection include corticosteroids and cyclosporin-A. However, prolonged use of these agents may be associated with serious complications, including

infection, cataract, glaucoma, and nephrotoxicity.<sup>2</sup> Therefore, development of more selective immunomodulatory strategies for the prevention of corneal graft rejection is desirable.

CD154, also known as CD40 ligand (CD40L), is a 39-kDa type II membrane glycoprotein and member of the tumor necrosis factor (TNF) superfamily. It is preferentially expressed on activated CD4<sup>+</sup> cells and mast cells. The counterreceptor for this ligand, CD40, is a 50-kDa integral membrane glycoprotein. It is found on a variety of antigen-presenting and mesenchymal cells, including B lymphocytes, dendritic cells, macrophages, monocytes, microglia, endothelial cells, and epithelial cells.<sup>3-5</sup> Over the past several years, the central role of CD40-CD154 interaction in mediating T-cell-mediated immune responses has been firmly established.<sup>3</sup> CD154-dependent activation of T cells occurs through signaling of CD40 at the level of antigen-presenting cells (APCs), which enhances requisite costimulatory pathways, including expression of B7.1/B7.2 (CD80/CD86).<sup>6</sup> Ligand of CD40 on APCs also triggers production of high levels of interleukin (IL)-12,<sup>7</sup> which is a potent stimulus for T-helper 1 (Th1) differentiation. Accordingly, it has been shown that blockade of the CD40-CD154 pathway is sufficient to induce tolerance to Th1-mediated contact hypersensitivity, and this is associated with inhibition of IL-12 mRNA expression and Th2 immune deviation.<sup>8</sup>

These data suggest that the CD40-CD154 pathway may serve as an ideal candidate for molecular intervention for immunomodulatory therapy in transplantation. In fact, consistent with its central role in cell-mediated immunity, blockade of CD40-CD154 interaction by anti-CD154 has been shown to prevent rejection of solid organ allografts, such as cardiac, renal, pancreatic islet, and skin grafts.<sup>9-11</sup> However, the effect of blocking the CD40-CD154 pathway on the fate of corneal transplants has not been determined. In this series of experiments, we studied the effect of systemic anti-CD154 monoclonal antibody (mAb) on the outcomes of normal-risk and high-risk allogeneic corneal grafts and explored whether this treatment can affect Th1-mediated allospecific delayed-type hypersensitivity (DTH) responses and the frequency of interferon (IFN)- $\gamma$ -producing T cells in the spleen and draining lymph nodes after transplantation.

## MATERIALS AND METHODS

### Mice and Anesthesia

Male BALB/c mice aged 8 to 10 weeks were purchased from Taconic Farms (Germantown, NY); B10.D2, BALB.b, and CB57L/6 mice of the same age were purchased from Jackson Laboratory (Bar Harbor, ME). Before all surgical procedures, each animal was deeply anesthetized by intraperitoneal injection of 3 to 4 mg of ketamine and 0.1 mg of xylazine. All animals were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

### Anti-CD154 Administration

Hamster anti-CD154 mAb IgG was purified from culture supernatant of MR1 hybridoma (American Type Culture Collection, Rockville, MD) by using a protein A fast-flow column and was kindly provided by Linda Burkly (Biogen, Cambridge, MA). Administration of anti-CD154 does

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From the Schepens Eye Research Institute, Department of Ophthalmology, Harvard Medical School, Boston, Massachusetts.

Supported by National Institutes of Health Grant NEI12963; Biogen; Eye Bank Association of America; Research to Prevent Blindness; and Fight For Sight.

Submitted for publication July 28, 2000; revised December 4, 2000; accepted December 20, 2000.

Commercial relationships policy: C (MRD), P (MRD), N (all others).

Corresponding author: M. Reza Dana, Laboratory of Immunology, Schepens Eye Research Institute, Harvard Medical School, 20 Staniford Street, Boston, MA 02114. dana@vision.eri.harvard.edu

not functionally delete antigen-specific Th cells in mice.<sup>12</sup> BALB/c mice were randomly selected to receive, in a masked fashion, either anti-CD154 or control hamster IgG immunoglobulin (Ig; Biogen) at a dose of 250  $\mu$ g/mouse by intraperitoneal injection. Because studies of other solid organ transplantations in animal models have reported that extended treatment with anti-CD154 is more effective in preventing graft rejection than is short-term treatment,<sup>11,13,14</sup> a sustained regimen of antibody therapy was applied in our studies on days -1, 0, and 1 and once weekly. The treatment was terminated at week 8, a time point at which donor-specific anterior chamber-associated immune deviation (ACAID) normally develops in hosts bearing accepted grafts, thereby sustaining long-term graft acceptance.<sup>15</sup>

### Induction of CNV and High-Risk Graft Beds

Suture-induced corneal neovascularization (CNV) is a standardized system of inducing neovascularization to create high-risk graft beds.<sup>16</sup> Three interrupted sutures (11-0 nylon, 50- $\mu$ m diameter needle; Sharp-point; Vanguard, Houston, TX) were placed in the central cornea of one eye of normal BALB/c mice. As described previously, neovascular growth into the normally avascular corneal stroma can be appreciated from the limbus as early as 3 days after suture placement. Neovessels occupy more than two quadrants of the central cornea after 14 days. These mice with neovascularized graft beds therefore served as high-risk recipients of orthotopic corneal transplants.

### Corneal Transplantation

BALB/c mice ( $n = 106$ ) were used as recipients of syngeneic (BALB/c,  $n = 10$ ), minor histocompatibility (H)-disparate (B10.D2,  $n = 48$ ), or major histocompatibility complex (MHC)-mismatched (BALB.b,  $n = 48$ ) corneal transplants. Mice bearing syngeneic grafts received anti-CD154 treatment. All other hosts were randomized to receive either anti-CD154 or control hamster Ig. Syngeneic grafts were transplanted to avascular (normal-risk) recipient beds; minor H-disparate and MHC-disparate grafts were transplanted to either avascular or neovascularized (high-risk) beds. Corneal transplantation was performed according to our well-established protocol.<sup>17</sup> Briefly on day 0, the central 2-mm area of the donor cornea was excised with Vannas scissors and secured in the host graft bed of 1.8-mm diameter with eight interrupted 11-0 nylon sutures (Sharp-point, Vanguard). Antibiotic ointment was applied to the corneal surface, and the eyelids were closed for 3 days with a tarsorrhaphy using 8-0 nylon sutures. All grafted eyes were examined 3 days after surgery, and transplant sutures were removed in all mice on day 7.

### Evaluation and Scoring of Orthotopic Corneal Transplant Rejection

All grafts were evaluated in a masked fashion for the signs of rejection by slit lamp biomicroscopy twice weekly over 8 weeks. The high-risk transplants were continuously observed until week 18 after the therapy was discontinued at week 8. At each time point, the grafts were scored for opacity. A previously defined and standardized scoring system was used to grade the degree of opacification from 0 to 5+ (0, clear graft; 1, minimal superficial opacity; 2+, mild stromal opacity with pupil margin and iris vessels visible; 3+, moderate stromal opacity with only pupil margin visible; 4+, intense stromal opacity with the anterior chamber visible; and 5+, maximal corneal opacity with total obscuration of the anterior chamber).<sup>17</sup> Grafts with an opacity score of 2+ or higher after 3 weeks were considered to be rejected, and grafts with an opacity score of 3+ or higher at 2 weeks that never cleared were also regarded as rejected.<sup>17</sup>

### Assessment of Donor-Specific DTH

Based on the finding that donor-specific DTH can be detected in high-risk recipients as early as 2 to 3 weeks after surgery,<sup>18,19</sup> we evaluated allospecific DTH responses in high-risk hosts ( $n = 5$ /group) bearing minor H-mismatched or MHC-disparate corneal grafts treated

with anti-CD154, 2 to 3 weeks after transplantation. The regimen of anti-CD154 or hamster control Ig treatment was the same as the aforementioned except that antibody administration was terminated 1 week before ear challenge. Irradiated (2000 rad) splenocytes ( $1 \times 10^6$ ) in 10  $\mu$ l of Hanks' balanced salt solution from BALB.b donors syngeneic with the corneal graft were injected into the right pinnae, as described previously.<sup>18</sup> BALB/c mice serving as positive controls were immunized by subcutaneous injection of  $10 \times 10^6$  BALB.b splenocytes 1 week before ear challenge. BALB/c mice serving as negative controls were challenged only with splenocytes but without prior immunization. At 24 and 48 hours after ear challenge, ear swelling was measured in a masked fashion with a low-pressure micrometer (Mitutoyo; MTI, Paramus, NJ). Ear-swelling responses are presented as mean  $\pm$  SE. Because results at 24 and 48 hours were similar, only 24-hour data are presented.

### ELISPOT Cytokine Measurement

The enzyme-linked immunospot (ELISPOT) assay was performed 2 weeks after high-risk transplantation of fully disparate corneal grafts. ELISPOT plates (Polyfiltronics; Rockland, MA) were coated with 4  $\mu$ g/ml of rat anti-mouse IFN- $\gamma$  (R4-6 A2) capturing mAb (PharMingen, San Diego, CA) in sterile phosphate-buffered saline (PBS) overnight. The plates were then blocked for 1.5 hours with sterile PBS containing 1% bovine serum albumin (BSA) and washed with sterile PBS. Responder cells, either  $8 \times 10^5$  draining lymph node cells or 0.5 to  $1 \times 10^6$  spleen cells, were then placed in each well in the presence or absence of mitomycin C-treated allogeneic splenocytes (as stimulator APCs; ratio 1:1). The cells were cultured at 37°C in 5% CO<sub>2</sub> for 42 hours. For detection of spots, 2  $\mu$ g/ml biotinylated rat anti-mouse IFN- $\gamma$  (XMG 1.2) mAb was used, followed by 1.5 hours of incubation with streptavidin D horseradish peroxidase (Vector, Burlingame, CA) diluted at 1:2000 in PBS with 0.025% Tween. All mAbs were purchased from PharMingen. After they were washed, the plates were developed using 0.8 ml of 3-amino-9-ethylcarbazole (AEC; Sigma, St. Louis, MO; 10 mg dissolved in 1 ml dimethylformamide) mixed with 24 ml of 0.1 M sodium acetate (pH 5.0), containing 12  $\mu$ l H<sub>2</sub>O<sub>2</sub>. The resultant spots were counted on a computer-assisted ELISPOT image analyzer (T Spot; Cellular Technology, Cleveland, OH).<sup>20</sup>

### Statistical Analysis

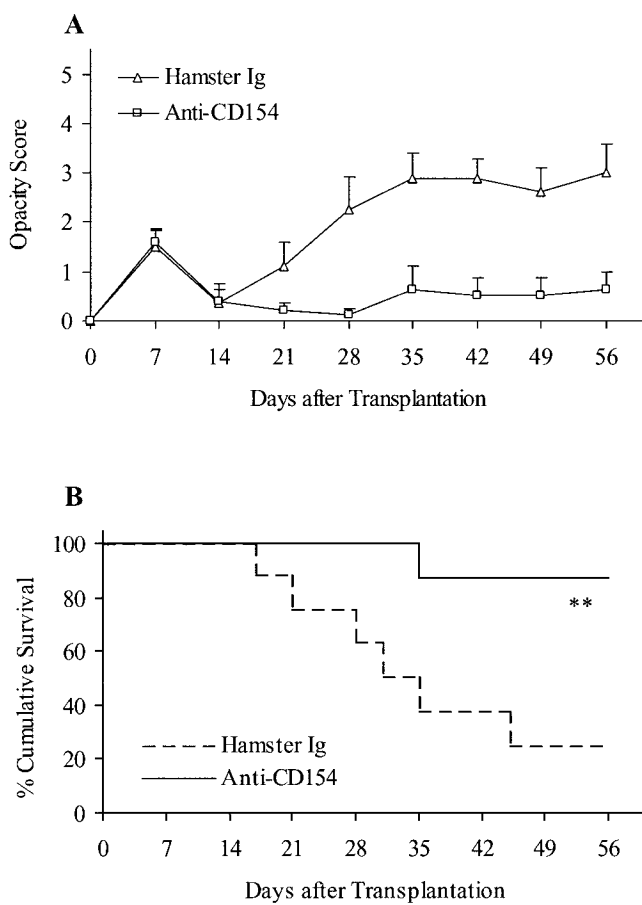
The rates of corneal graft survival were plotted as Kaplan-Meier survival curves and compared by using the log rank (Mantel-Cox) test. DTH data are presented as mean  $\pm$  SE and were compared by using analysis of variance (ANOVA). Statistical significance was defined as a  $P < 0.05$ .

## RESULTS

MHC-disparate and minor H-disparate corneas were grafted to BALB/c recipients randomized to receive either hamster mAb or anti-CD154 therapy. Mice bearing syngeneic BALB/c grafts treated with systemic anti-CD154 served as treatment control animals. Syngeneic grafts had a survival rate of 100% at 8 weeks. There were no primary graft failures in any of the transplants.

### Fate of Normal-Risk Corneal Grafts

Minor H-disparate grafts treated with hamster Ig exhibited vigorous and prompt rejection (Fig. 1), beginning as early as 17 days after transplantation. The cumulative survival rates of this group at 4 and 8 weeks were 64% and 25%, respectively. In contrast, minor H-disparate grafts treated with anti-CD154 displayed significantly increased survival. The only allograft rejection in this group occurred on day 35. The cumulative survival rates in this group at 4 and 8 weeks were 100% and 87%, respectively—significantly higher than rates seen among the hamster mAb-treated control mice ( $P = 0.0087$ ). The data



**FIGURE 1.** Fate of normal-risk minor H-disparate corneal transplants. BALB/c mice ( $n = 10/\text{group}$ ) received corneal transplants from B10.D2 mice and were randomized to receive anti-CD154 or control hamster Ig. Graft opacification was scored clinically (A), and graft survival data are presented as Kaplan-Meier survival curves (B). Anti-CD154 treatment promoted the survival of minor H-disparate grafts (\*\* $P = 0.0087$ ).

demonstrate that systemic anti-CD154 administration can profoundly reduce the rejection rate of minor H-disparate corneal allografts.

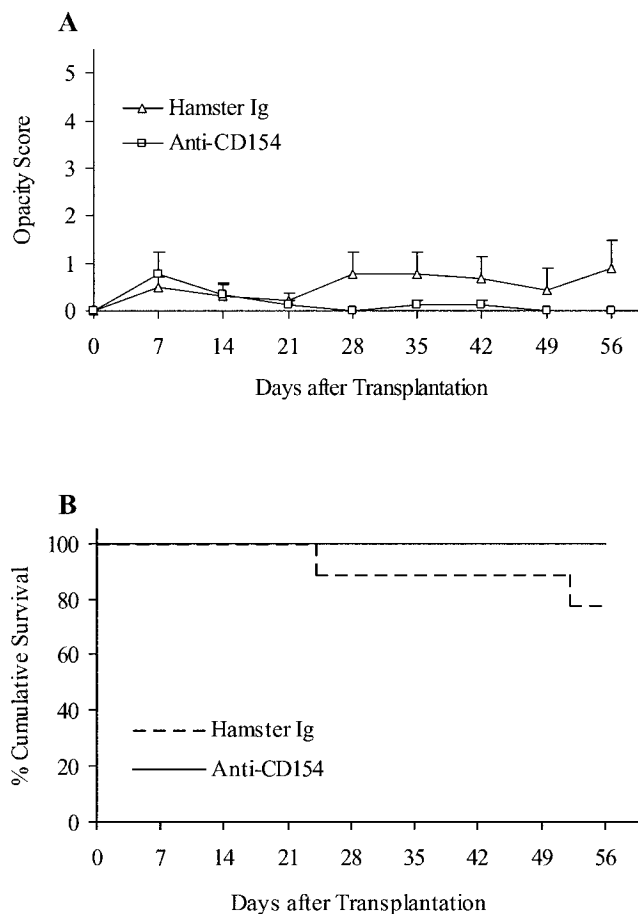
The results in MHC-disparate transplants (Fig. 2) showed survival rates of 89% and 78% in hamster Ig-treated mice at 4 and 8 weeks, respectively. In contrast, grafted hosts treated with anti-CD154 had universal survival of the allogeneic grafts for the entire treatment period. However, because of the relatively high rate of acceptance of MHC-disparate grafts among control hosts, the increased rate of graft survival among hosts treated with anti-CD154 did not reach statistical significance ( $P = 0.177$ ). This high survival rate of MHC-disparate grafts, even among untreated control animals, is consistent with previous data suggesting that minor alloantigens play a more significant role in corneal allograft rejection.<sup>17</sup>

### Fate of High-Risk Corneal Grafts

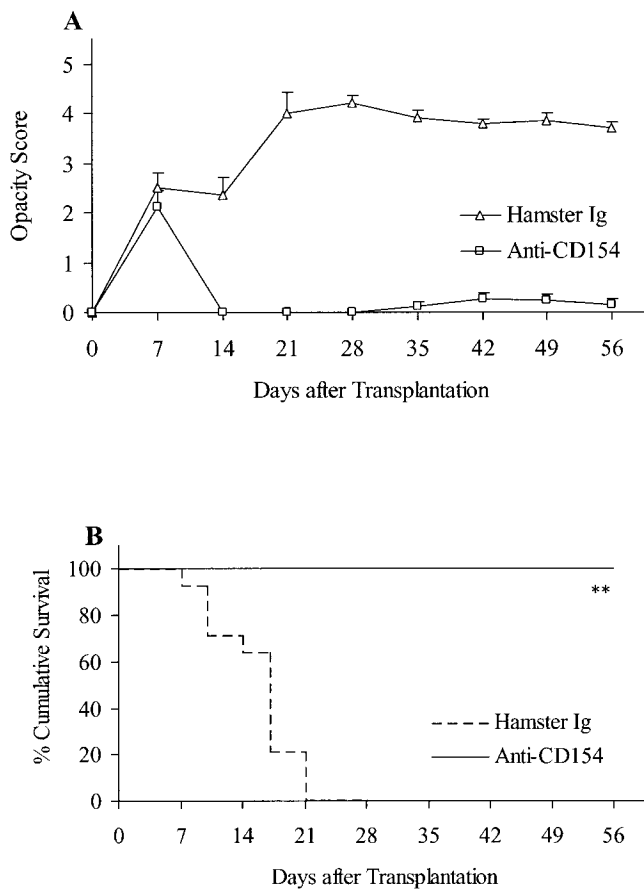
Corneal allografts placed in neovascularized high-risk beds of recipient mice experience more rapid rejection and markedly lower survival than do grafts placed in avascular normal-risk beds in both rodents and humans.<sup>21</sup> Therefore, anti-CD154 therapy was extended to include high-risk corneal transplants. Control animals receiving high-risk minor H-disparate grafts exhibited swift rejections. The first rejection occurred at day 7, and all grafts were rejected by week 4. In contrast, 100% of

anti-CD154-treated grafts survived during the 8-week period of treatment ( $P = 0.0001$ ; Fig. 3). Because MHC-disparate corneal grafts transplanted into normal avascular beds have a high acceptance rate even in control animals, the effect of anti-CD154 was further tested on MHC-disparate grafts transplanted into high-risk vascularized beds that exhibit a swift rejection in control hosts.<sup>21</sup> Among control hamster Ig-treated hosts, only 27% and 13% of grafts survived at 4 and 8 weeks, respectively, after transplantation, with a majority of the grafts being rejected at 17 days. In contrast, anti-CD154 therapy dramatically improved graft survival to 92% at both 4 and 8 weeks ( $P = 0.0002$ ), with only one graft rejected at 21 days (Fig. 4). These results demonstrate that systemic anti-CD154 treatment can prevent corneal graft rejection in high-risk transplantation.

To determine whether anti-CD154 acts as a transiently immunosuppressive agent or whether it can promote long-term graft acceptance even after discontinuation, mice that had received high-risk grafts were monitored after the antibody treatment was discontinued. Among recipients of high-risk minor H-disparate grafts, all transplants survived for an additional 4 weeks after cessation of anti-CD154 treatment. However, rejections occurred gradually thereafter, and the graft survival rate decreased from 100% at week 12 to 60% at week 15. No additional rejections were observed among



**FIGURE 2.** Fate of normal-risk MHC-disparate corneal transplants. BALB/c mice ( $n = 10/\text{group}$ ) received corneal transplants from BALB.b mice and were randomized to receive anti-CD154 or control hamster Ig. Graft opacification was scored clinically (A), and graft survival data are presented as Kaplan-Meier survival curves (B). There was universal survival of MHC-disparate allografts in hosts treated with anti-CD154 ( $P = 0.177$ ).



**FIGURE 3.** Fate of high-risk minor H-disparate corneal transplants in BALB/c mice ( $n = 14/\text{group}$ ) that received corneal grafts from B10.D2 mice and were randomized to receive anti-CD154 or hamster Ig. Graft rejection was scored clinically (A), and graft survival data are presented as Kaplan-Meier survival curves (B). Anti-CD154 therapy enhanced the survival of minor H-disparate grafts in high-risk transplantation (\*\* $P = 0.0001$ ).

these grafts for the duration of follow-up (Fig. 5). In contrast, cessation of therapy among high-risk MHC-disparate grafts led to only one additional graft rejection (week 9). No additional rejections were observed for the duration of follow-up (Fig. 6). In the aggregate, the data suggest that although anti-CD154 treatment is profoundly effective in suppressing allograft rejection, even in high-risk transplantation, its efficacy is diminished after cessation of therapy, particularly among minor H-disparate grafts.

### Donor-Specific DTH

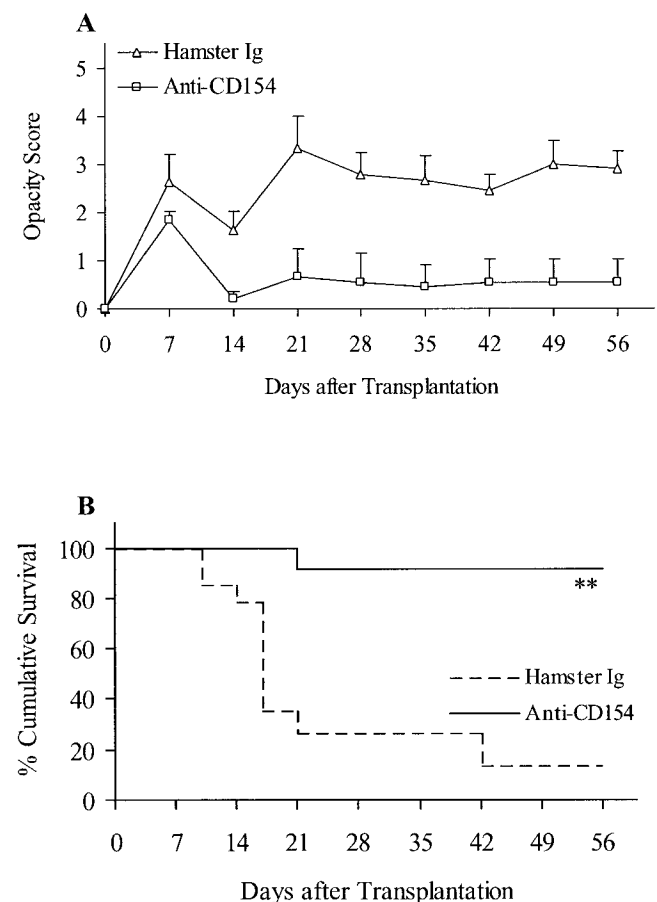
Donor-specific DTH was evaluated in BALB/c mice after high-risk corneal transplantation. Control hosts treated with hamster Ig mounted a vigorous ear-swelling response to splenocytes from B10.D2 (minor H-disparate, Fig. 7A), or BALB.b (MHC-disparate, Fig. 7B) mice. Although not as vigorous as that in DTH-positive controls, the allospecific DTH response among grafted animals treated with control Ig was more intense than that in naïve animals. In contrast, anti-CD154-treated hosts exhibited a significant decrease in the degree of ear swelling compared with hamster Ig-treated control mice ( $P < 0.05$ ), suggesting that anti-CD154 therapy leads to suppression of Th1 type responses.

### IFN- $\gamma$ Profile of Corneal Graft Recipients in Response to Donor Alloantigen

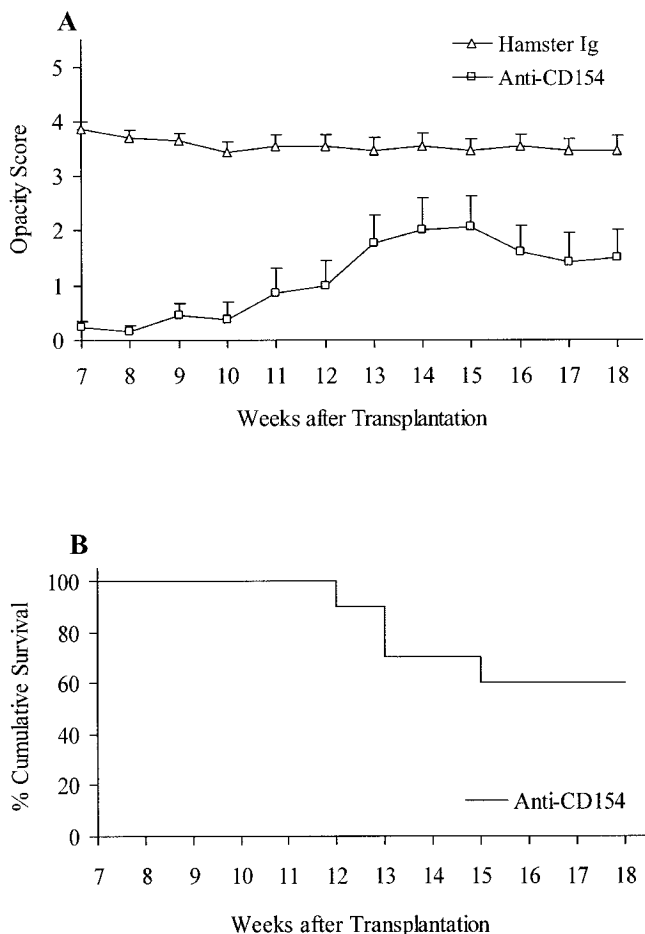
A highly sensitive ELISPOT technique was used to determine the frequency of IFN- $\gamma$ -producing alloreactive T cells in the cervical draining lymph nodes and in the spleen 2 weeks after corneal transplantation. As shown in Figure 8A, the frequencies of alloreactive IFN- $\gamma$ -producing recipient cells in the draining lymph nodes was dramatically lower in anti-CD154-treated hosts than in hamster Ig-treated hosts. Similar result was also obtained for the splenocytes (Fig. 8B).

### DISCUSSION

Our data demonstrate that the incidence of corneal allograft rejection was dramatically reduced in recipients with intermittently administered anti-CD154 mAb as a sole therapy. The results highlight the important function of the CD40-CD154 costimulatory pathway in the immunobiology of corneal transplantation as reflected by the nearly universal acceptance of allografts in both normal-risk and high-risk hosts receiving anti-CD154 therapy. Because work in a number of laboratories, including ours, has suggested that several facets of alloimmunity differ between MHC- and minor H-mismatched grafts, we tested the effect of CD40-CD154 blockade separately on MHC- and minor H-mismatched allografts. Our findings indicate that



**FIGURE 4.** Fate of high-risk MHC-disparate corneal transplants in BALB/c mice ( $n = 14/\text{group}$ ) that received corneal grafts from BALB.b mice and were randomized to receive anti-CD154 or hamster Ig. Graft rejection was scored clinically (A), and graft survival data are presented as Kaplan-Meier survival curves (B). Anti-CD154 therapy enhanced the survival of minor H-disparate grafts in high-risk transplantation (\*\* $P = 0.0002$ ).



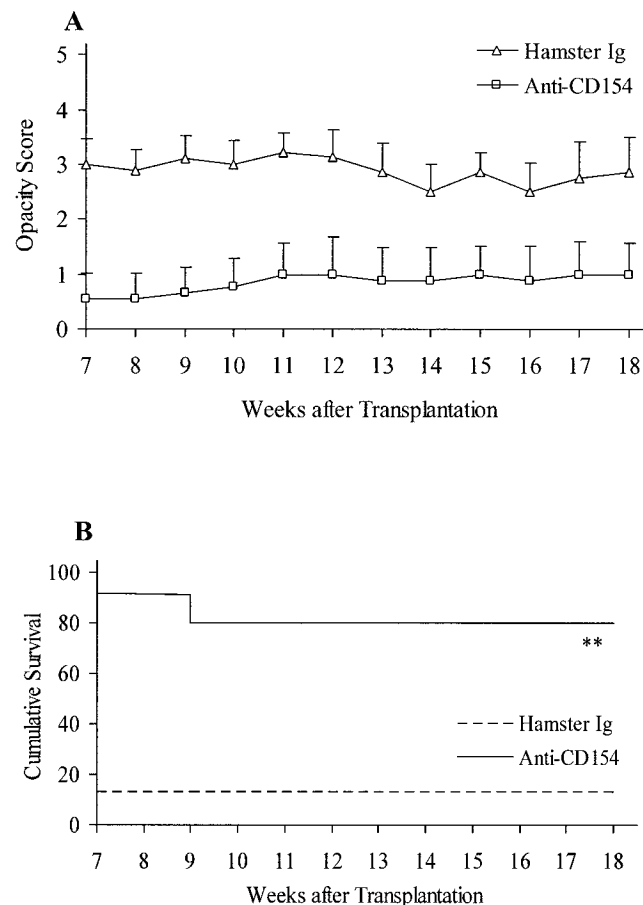
**FIGURE 5.** Fate of high-risk minor H-disparate corneal transplants in mice ( $n = 14/\text{group}$ ) after anti-CD154 treatment was discontinued at week 8 after transplantation. Graft rejection was scored clinically (A), and graft survival data are presented as Kaplan-Meier survival curves (B). Anti-CD154 therapy did not completely inhibit delayed corneal allograft rejection in minor H-disparate high-risk recipients.

the CD40-CD154 costimulatory pathway is involved in graft rejection of both allocombinations.

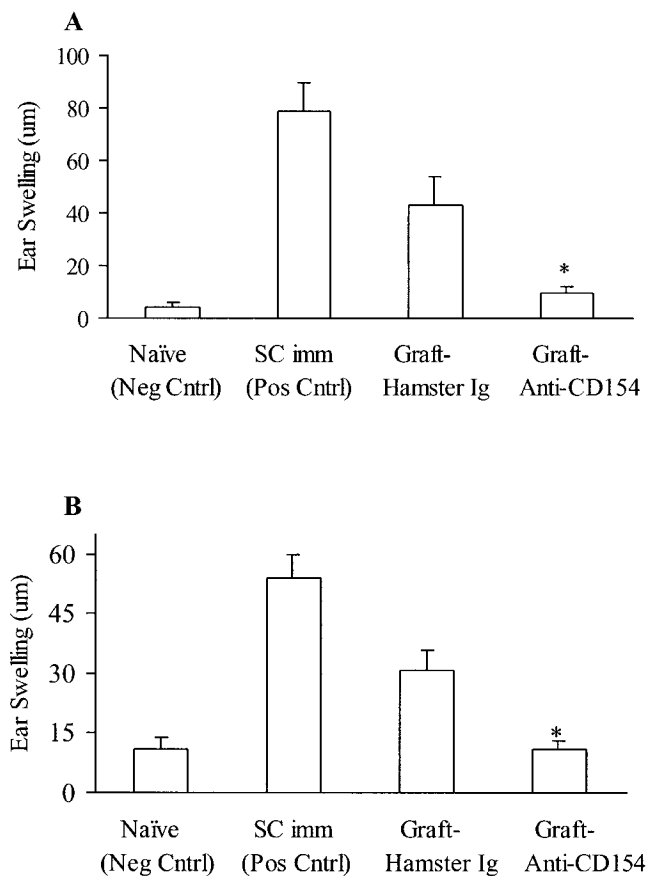
Anti-CD154 monotherapy has been shown to effectively prevent acute rejection of cardiac, renal, and pancreatic islet allografts in both primates and rodents.<sup>9-12</sup> Our current findings in corneal transplants are in agreement with these studies. The reported timing and duration of anti-CD154 administration in the previously reported studies is variable, ranging from only once at surgery to once or twice weekly for 2 to 7 weeks followed by once monthly for maintenance. In the aggregate, it appears that the timing of initial administration is critical. When treatment is delayed until 5 days after surgery in the mouse model of cardiac transplantation, no prolongation of graft survival has been observed.<sup>10</sup> Moreover, an extended regimen is significantly more effective in preventing graft rejection than brief (less than 2 weeks) perioperative treatment. For example, a preliminary study has shown that administration of anti-CD154 only during the first postoperative week delays corneal graft rejection but improves overall graft survival only minimally.<sup>22</sup> The disparity between the results of this report and ours is probably due to the different regimens used. Because sensitization to corneal allografts occurs within the first several weeks after transplantation,<sup>18,19</sup> coverage of the host with anti-CD154 at least during this period appears to be critical in promoting graft success.

The exact mechanism of action of anti-CD154 monotherapy in the prevention of corneal allograft rejection remains to be fully elucidated. Our findings indicate that the Th1-mediated allospecific DTH response is suppressed in animals receiving anti-CD154 treatment. To distinguish whether anti-CD154 therapy suppresses the induction or expression of the DTH response, we have evaluated the effect of anti-CD154 on allospecific DTH response in BALB/c mice presensitized by subcutaneous injection of B6 spleen cells. The mice were given either anti-CD154 antibody or hamster Ig 1 week after immunization and 1 week before ear challenge. We found that anti-CD154 antibody-treated mice mounted a strong DTH response that was comparable to that in hamster Ig-treated mice as well as that in DTH-positive control animals. Thus, the data suggest that anti-CD154 treatment is effective through its inhibitory function on induction of the DTH response. Because alloreactive responses of the DTH type correlate strongly with graft rejection, as reported by many laboratories,<sup>17,23-25</sup> anti-CD154 may therefore prolong corneal graft survival through suppression of Th1-type sensitization.

In addition to testing for the effect of anti-CD154 therapy on induction of allospecific DTH, we additionally evaluated the effect of such treatment on the frequency of IFN- $\gamma$ -producing T cells in the hosts. Consistent with the DTH data, we observed a significant reduction of IFN- $\gamma$ -secreting Th1 cells in hosts of grafts receiving anti-CD154 treatment. Similar to our data, it has been shown that the profile of cytokine expression in murine



**FIGURE 6.** Fate of high-risk MHC-disparate corneal transplants in mice ( $n = 14/\text{group}$ ) after anti-CD154 treatment was discontinued at week 8 after transplantation. Graft rejection was scored clinically (A), and graft survival data are presented as Kaplan-Meier survival curves (B). Only one rejection occurred after cessation of anti-CD154 therapy (\*\* $P = 0.0002$ ).



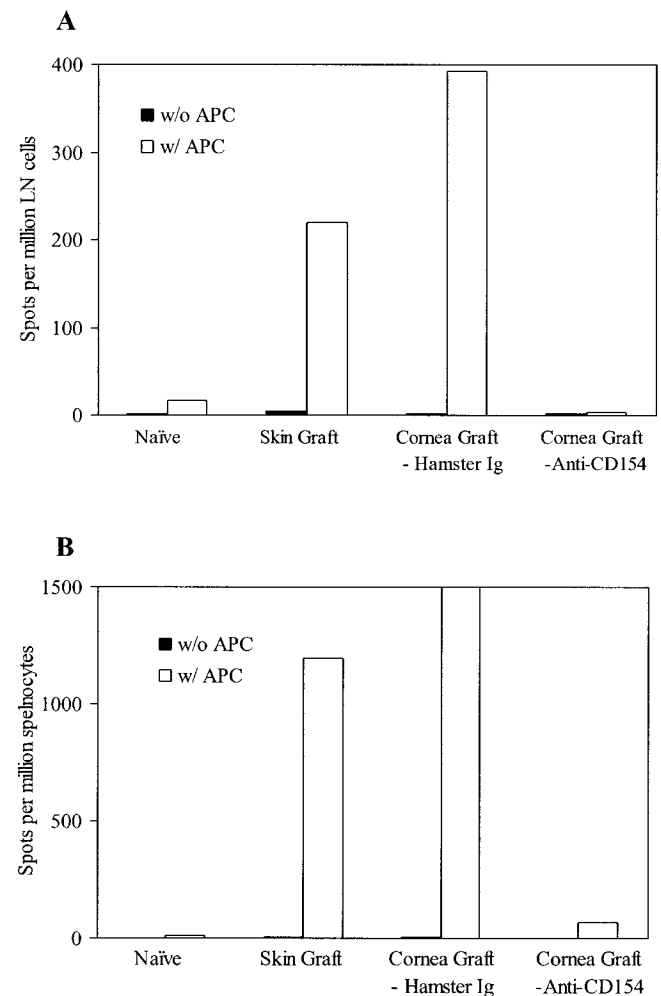
**FIGURE 7.** Donor-specific DTH in BALB/c mice bearing orthotopic of B10.D2 minor H-disparate corneal allografts (A) or BALB.b MHC-mismatched grafts (B) 2 to 3 weeks after high-risk transplantation. Ear swelling of naïve (negative control), subcutaneously primed (positive control), control hamster Ig-treated, and anti-CD154-treated mice were measured by a micrometer 24 and 48 hours after ear challenge. Anti-CD154 therapy significantly inhibited the degree of ear swelling compared with positive control animals or mice receiving control hamster Ig treatment (\* $P < 0.05$ ).

cardiac allografts changes from a Th1-biased (IFN- $\gamma$  and IL-2) to a Th2-biased (IL-4 and IL-10) pattern in anti-CD154-treated animals.<sup>26</sup> Although our data in regard to CD40-CD154 blockade and suppression of the IFN- $\gamma$  response support the *in vivo* observation of suppressed DTH, we cannot at this point comment on the Th2 cytokine response. Because deviation away from a Th1 response in the corneally grafted host prevents corneal graft rejection,<sup>27</sup> it would be of additional interest to examine the Th2 cytokine response in these anti-CD154-treated hosts, or to block Th2 cytokine activity, such as IL-4 or IL-10, to observe whether anti-CD154 treatment is still effective in preventing graft rejection.

Although anti-CD154 suppresses the induction of allosensitization, studies suggest that inhibition of allosensitization alone may not be sufficient to maintain long-term graft acceptance in high-risk eyes.<sup>28</sup> In fact, contrary to studies on renal and pancreatic islet transplantation in which long-term graft survival has been reported even after cessation of anti-CD154 therapy,<sup>9,11</sup> 40% of the accepted high-risk grafts in our study were rejected 4 weeks after withdrawal of therapy among the minor H-disparate grafts. The reasons for this observation remain unclear. In some CD4<sup>+</sup>-deficient mice, fully mismatched corneal allografts are rejected within 10 weeks of engraftment<sup>29</sup> or undergo delayed rejection after long-term acceptance.<sup>30</sup> The CD4<sup>+</sup> T-cell knockout mice that have re-

jected allogeneic grafts do not generate significant DTH responses,<sup>29</sup> suggesting a mechanism involving CD8<sup>+</sup> T cells. Based on these findings, it is possible that donor-specific CD8<sup>+</sup> T cells will eventually emerge as effectors of rejection of corneal allografts. Of note, unlike our results with minor H-disparate high-risk grafts, the majority of high-risk MHC-disparate transplants enjoyed prolonged acceptance, even after the therapy was withdrawn. This may be due to the less critical role of MHC disparity in corneal allograft rejection.<sup>21</sup>

It is important to emphasize that we cannot state with certainty whether the prolongation of graft survival by the treatment was due entirely to suppressed allosensitization or is also due to early acquisition of allospecific tolerance. Previous



**FIGURE 8.** Frequency of IFN- $\gamma$ -producing donor-specific T cells in the draining lymph nodes (LN; A) and the spleen (B) of high-risk graft recipients after allogeneic corneal transplantation. High-risk BALB/c mice received transplants of fully mismatched C57BL/6 allogeneic corneas. Recipient draining cervical lymph nodes and spleens were removed 2 weeks after transplantation. The frequencies of IFN- $\gamma$ -producing T cells in naïve mice, hamster Ig-treated hosts, anti-CD154-treated recipients, and BALB/c mice bearing fully mismatched C57BL/6 skin grafts were then determined using ELISPOT assay. *Filled bars*: Control animals, demonstrating nonspecific response in which host responder cells were not incubated with donor APCs; *open bars*: represent allospecific cytokine response as host responder cells were stimulated with donor APCs. The experiments were repeated three times with similar results. As shown in this representative graph, anti-CD154-treated mice exhibited significantly lower frequencies of IFN- $\gamma$ -producing alloreactive T cells than did hamster Ig-treated recipients.

reports indicate that mice bearing accepted corneal allografts for 8 weeks or more show development of allospecific ACAID,<sup>31</sup> which is one form of tolerance. We have found that anti-CD154-treated acceptors of high-risk grafts acquire ACAID to donor alloantigens at week 18, whereas hamster Ig-treated control mice universally do not to acquire ACAID at the same time point (data not shown). However, we cannot address at this time whether blockade of the CD40-CD154 costimulatory pathway induces early development of ACAID, even before 8 weeks after surgery.

The currently available preventive regimens for corneal transplant rejection are associated with significant complications, and it thus would be desirable to devise intervention strategies that can prolong graft survival by specifically targeting molecular mediators involved in generation of the alloimmune response. A variety of successful experimental strategies have been developed including induction of tolerance to donor corneal cells,<sup>32</sup> macrophage depletion,<sup>33</sup> deviation of recipient immune systems toward Th2 response,<sup>27</sup> and intervention with the function or expression of adhesion molecules, cytokines, or T cells.<sup>34-37</sup>

Although further studies are needed to determine whether treatment with anti-CD154 can alter induction of tolerance to transplantation antigens and whether local (ocular) application of anti-CD154 can similarly promote allograft survival, our data demonstrating near-universal graft acceptance regardless of the degree of allodisparity or risk indicate that blockade of the CD40-CD154 costimulatory pathway holds significant promise as an effective modality for promoting corneal transplant survival. However, because it is suspected that CD8<sup>+</sup> T cells may mediate delayed corneal allograft rejection, anti-CD154 therapy may not prevent delayed corneal rejection, because CD154 is not an important costimulatory molecule for CD8<sup>+</sup> T cell activation.<sup>36,39</sup> Accordingly, the long-term prevention of graft rejection may require alternative strategies after cessation of anti-CD154 therapy.

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