Effect of LFA-1 and ICAM-1 Antibody Treatment on Murine Corneal Allograft Survival

YuGuang He,* Jessamee Mellon,* Rajendra Apte,† and Jerry Y. Niederkorn*

Purpose. To examine the effect of anti-LFA-1 and anti-ICAM-1 antibody treatment on orthotopic corneal graft survival in a mouse model.

Methods. Anti-LFA-1 and anti-ICAM-1 antibodies were administered intraperitoneally before and shortly after orthotopic corneal transplantation. Grafts were observed by biomicroscopy, and survival times were determined. Cytotoxic T lymphocyte (CTL) and delayed-type hypersensitivity (DTH) responses to donor alloantigens were assessed at selected times after grafting.

Results. Administration of anti-LFA-1 antibody reduced the incidence of graft rejection from 90% in untreated donors to 47% in anti-LFA-1 treated mice. By contrast, treatment with anti-ICAM-1 antibody alone did not reduce the incidence of rejection, although it prolonged graft survival time. Both CTL and DTH responses to donor alloantigens were severely depressed in hosts treated with either anti-LFA-1 or anti-ICAM-1 antibody. However, neither anti-ICAM-1 nor anti-LFA-1 antibody treatment prevented the rejection of orthotopic corneal grafts in previously immunized mice.

Conclusions. Anti-ICAM-1 antibody does not promote graft survival even though it impairs CTL and DTH responses to donor alloantigens. By contrast, anti-LFA-1 antibody can significantly reduce the incidence of orthotopic corneal graft rejection and prevent the induction of normal allospecific CTL and DTH responses. Although anti-LFA-1 antibody is effective if given prophylactically, it is ineffective at preventing corneal graft rejection in previously immunized hosts. Invest Ophthalmol Vis Sci. 1994; 35:3218-3225.

Keratoplasty is one of the oldest, most common, and most successful forms of organ transplantation.1 Approximately 40,000 corneal transplants are performed each year in the United States.2 Despite a success rate that often approaches 90%, a significant number of corneal grafts fail because of immunologic rejection.2-4 Thus, understanding the mechanisms of corneal graft rejection and developing improved immunosuppressive strategies could have a major impact on promoting corneal allograft survival and restoring vision.

Studies on allogeneic organ transplantation in experimental animals have demonstrated that leukocytes traffic into and out of allografts during the acute stage of rejection.5 The initial step in graft invasion by recipient cells is mediated by cell adhesion molecules, such as leukocyte function antigen-1 (LFA-1) and intercellular adhesion molecule-1 (ICAM-1), members of the integrin and immunoglobulin supergene families.6-7 LFA-1 is required for optimizing T-cell function in vitro, such as generation of cytotoxic T lymphocytes (CTL), activation of antigen receptors on T cells, and lymphokine production by helper T cells after antigen presentation.6-7 ICAM-1 participates in T-cell migration and infiltration in some autoimmune diseases.8 Thus, it is speculated that LFA-1/ICAM-1 cellular interaction is critically involved in allograft rejection, as well as in inflammatory responses.

Monoclonal antibodies directed against cell adhesion molecules are potential agents for preventing graft rejection and inflammation. Several laboratories have reported that systemic administration of anti-LFA-1 and anti-ICAM-1 monoclonal antibodies leads to specific tolerance and long-term acceptance of heteropic cardiac allografts in rodents9,10 and promo-
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Antibody treatment also ameliorated experimental autoimmune diseases, including experimental autoimmune uveitis and adjuvant-induced arthritis. In the present study, we employed a well-characterized mouse orthotopic corneal transplantation model to study the efficacy of anti-LFA-1 and anti-ICAM-1 antibody treatments in promoting corneal allograft survival in mice.

MATERIALS AND METHODS

Animals

Female C3H (H-2k) and CB6F1 (H-2b/d) mice were purchased from the Jackson Laboratories (Bar Harbor, ME) and used between the ages of 2 and 8 months. All experimental animals were handled in accordance with the ARVO Resolution on the Use of Animals in Research.

Orthotopic Corneal Transplantation

Full-thickness penetrating C3H corneal grafts (2.5 mm diameter) were transplanted orthotopically onto anesthetized CB6F1 mice using a procedure previously described by She et al and modified by He et al. Mice were anesthetized with an intraperitoneal injection of sodium pentobarbital (1 to 2 mg/mouse; Abbott Laboratories, Chicago, IL). Proparacaine was used as a topical anesthetic (Alcon Laboratories, Fort Worth, TX). Both the donor graft and the recipient graft bed were scored with 2.5 mm and a 2.0 mm diameter trephines, respectively (Storz Instrument, St. Louis, MO) before removal of the corneal button using a vannas scissors (Storz). The donor graft was sewn into place using 12 interrupted 11-0 nylon sutures and a 50-µm diameter needle (Sharpoint, Vanguard, Houston, TX). Sutures were completely removed 7 to 10 days later. Topical antibiotic (tobramycin; Alcon) was applied twice a week after surgery. No immuno-suppressive drugs were used.

Clinical Observations

Grafted eyes were examined with a slit-lamp biomicroscope at least twice a week throughout the entire study period. Graft opacity, edema, and neovascularization were scored as minimal, moderate, or severe, as previously described. If all three parameters became moderate or severe more than 7 days after transplantation, the graft was recorded as rejected on that day. Any host that developed complications such as cataract, anterior chamber loss, iris synechiae, or infection, was excluded from the study. Mean survival time was calculated for each group, and the Mann-Whitney test was used to determine the statistical significance of the results.

Monoclonal Antibodies

The M17/4.2 hybridoma (TIB217, American Type Culture Collection, Rockville, MD) secretes a rat anti-murine LFA-1 α subunit specific IgG2a, isotype immunoglobulin. The YN1/1.7.4 (CRL1878 American Type Culture Collection) secretes a rat monoclonal antibody (IgG2b) that reacts with a murine ICAM-1. Both hybridomas were propagated in nude mice, and the monoclonal antibodies were isolated and purified from ascites fluid as previously described. Experimental mice were grafted orthotopically on day 0, and the designated antibodies were given intraperitoneally at a dose of 0.10 to 0.20 mg/mouse on days −2, −1, 0, 2, 4, 7.

Flow Cytometry

The efficacy of the in vivo antibody treatments was assessed by flow cytometry. LFA-1 and ICAM-1 expressions on splenocytes from grafted mice treated with either monoclonal antibody were analyzed by fluorescein-activated cell sorter (FACS). Indirect immunofluorescent staining was performed with either M17/4.2 (anti-LFA-1) or YN1/1.7.4 (anti-ICAM-1) as a primary antibody, FITC-labeled goat anti-rat IgG as a secondary antibody. Background staining with secondary antibody only was set at 1% of total cells. GK1.5 (rat anti-mouse CD4) and YTS169.4 (rat anti-mouse CD8) were also used as primary antibodies to analyze the influence of in vivo anti-LFA-1 and anti-ICAM-1 mAb treatment on these T-cell populations.

Cell-Mediated Cytotoxicity Assay

Selected CB6F1 recipient mice were killed either during the early stage (day 7 or day 14) or the late stage (days 35 to 45) after transplantation. Single-cell suspensions were prepared from spleens removed from individual animals and were used as effector cells in conventional in vitro cell-mediated cytotoxicity assays as previously described. Experimental and control effector lymphocytes were boosted in vitro for 48 hours at 37°C with gamma-irradiated (5,000 cGy) C3H stimulator spleen cells. After in vitro boosting, the effector cells were washed and resuspended in complete medium, and 100 µl of the various concentrations of the effector cell suspensions were added to round-bottomed microtiter plates. Chromium-labeled C3H ConA blasts (1 × 10⁶ cells) were added to the various wells to produce effector to target ratios ranging from 100:1 to 12.5:1. Plates were centrifuged at 100g for 2 minutes and incubated at 37°C for 4 hours in a humidified 5% CO₂ atmosphere. Plates were then centrifuged, 100 µl of each supernatant was collected, and the counts per minute were determined by counting the specimens in a gamma counter (Tra-
Analytical, Atlanta, GA). Cytotoxicity was calculated according the formula:

\[
\% \text{ Specific cytotoxicity} = \frac{\text{Exp. cpm} - \text{spontaneous release cpm}}{\text{Max. release} - \text{spontaneous release cpm}} \times 100
\]

The statistical significance among the various groups was determined by Student’s t-test.

**Delayed-Type Hypersensitivity Assay**

Delayed-type hypersensitivity (DTH) responses to allografts were measured by a conventional footpad swelling assay. Experimental CB6F1 mice received corneal grafts on day 0 and the designated mAb injections on days -2, -1, 0, 2, 4, 7. Control groups consisted of naive age-matched CB6F1 mice that were not grafted (negative control) and their counterparts that were grafted heterotopically but not treated with mAb (positive control). Footpads were challenged and measured for DTH responses either at the early stage (day 14) or the late stage (day 45) after corneal transplantation. Both hind footpads of each mouse were measured with an engineer’s micrometer (Mitutoyo, Tokyo, Japan) immediately before footpad challenge. An eliciting dose of $1 \times 10^7$ gamma-irradiated (3,000 cGy) C3H splenocyte suspensions in 25 μl of Hank’s balanced salt solution (HBSS) was injected into the subcutaneous tissue of the right hind footpad. The left hind footpad served as a negative control and received 25 μl of HBSS without splenocytes. Both footpads were measured 24 hours later, and the difference in footpad swelling size was used as a measure of DTH. Results are expressed as specific footpad swelling, which equals:

\[
[(24\text{-hour right hind foot measurement} - 0\text{-hour right hind foot measurement}) - (24\text{-hour left hind foot measurement} - 0\text{-hour left hind foot measurement})] \times 10^{-4} + \text{SE in inches.}
\]

Student’s t-test was used to evaluate the statistical significance of the results.

**Heterotopic Corneal Transplantation**

To determine the effect of LFA-1 and ICAM-1 mAbs in preventing corneal allograft rejection in high-risk hosts, full-thickness C3H corneal grafts along with the peripheral limbus (total 3.0 to 3.5 mm diameter) were transplanted heterotopically onto vascularized subdermal graft beds on the lateral thorax of CB6F1 mice as previously described. Two corneas were grafted to each experimental mouse. Plaster casts were left in place until 14 days after transplantation.

**RESULTS**

**Effect of Anti-LFA-1 and Anti-ICAM-1 on Corneal Graft Survival**

The present donor-host combination represented a complete mismatch at the major histocompatibility complex as well as at multiple minor histocompatibility loci. In this donor-host combination, the incidence of graft rejection in untreated normal hosts was 90% (Fig. 1). Although systemic treatment with anti-ICAM-1 mAb prolonged the survival time of corneal grafts, it did not reduce the incidence of rejection. By contrast, treatment with anti-LFA-1 mAb resulted in a significant reduction in the incidence of rejection as well as a prolongation of the mean survival time compared to the untreated mice. However, combined treatment with LFA-1 and ICAM-1 mAbs did not reduce the rejection rate any further than anti-LFA-1 mAb alone (Fig. 1).

To exclude the possibility that the rejection rate might be reduced further by a more aggressive mAb treatment, one group of animals (n = 5) was injected with both anti-LFA-1 and anti-ICAM-1 mAbs weekly after the initial treatment protocol until 8 weeks after grafting or until rejection occurred. The incidence of
TABLE 1. Effect of Anti-LFA-1 and Anti-LFA-1 on Corneal Graft Survival

<table>
<thead>
<tr>
<th>Group</th>
<th>No. Rejected/No. Grafted</th>
<th>Mean Survival Time ± SD</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>9/10 (90%)</td>
<td>17.2 ± 2.7*</td>
<td></td>
</tr>
<tr>
<td>aICAM-1</td>
<td>8/8 (100%)</td>
<td>25.3 ± 3.3</td>
<td></td>
</tr>
<tr>
<td>aLFA-1</td>
<td>8/17 (47%)</td>
<td>23.9 ± 5.8</td>
<td></td>
</tr>
<tr>
<td>aLFA-1 + aICAM-1</td>
<td>9/18 (50%)</td>
<td>28.6 ± 8.3</td>
<td></td>
</tr>
<tr>
<td>aLFA-1 + aICAM-1† (preimmunized)</td>
<td>5/5 (100%)</td>
<td>17.0 ± 4.8</td>
<td></td>
</tr>
<tr>
<td>aLFA-1 + aICAM-1§ (aggressive protocol)</td>
<td>3/5 (60%)</td>
<td>21.0 ± 11.5</td>
<td></td>
</tr>
<tr>
<td>Second graft†</td>
<td>8/8 (100%)</td>
<td>14.9 ± 3.8</td>
<td></td>
</tr>
</tbody>
</table>

* MST calculated only for those animals in which grafts underwent rejection. Hosts with long-term grafts were not used for MST calculations.
† P value compared to untreated group (line #1).
‡ Mice were preimmunized with two heterotopic corneal allografts 14 days before treatment with anti-LFA-1 + anti-ICAM-1 mAbs. Orthotopic corneal allografts were transplanted 2 days after initiation of antibody treatment. Antibodies were administered as described in Materials and Methods.
§ Combined treatment with anti-LFA-1 + anti-ICAM-1 administered on days −2, −1, 0, 2, 4, 7, and at 7-day intervals until day 45 or until graft rejection.
¶ P value comparing “aggressive protocol” mAb treatment group with “normal protocol” mAb treatment group (line #4).
† Fate of orthotopic corneal allografts transplanted to the left eyes of anti-LFA-1-treated mice bearing long-term (i.e., >45 day survival) orthotopic corneal allografts in the right eyes.

Rejection and the mean survival time in these aggressively treated hosts were not significantly different from those found in animals injected with mAb using the original protocol (P > 0.05) (Table 1).

Effect of mAb Treatment on the Induction of Allospecific CTL and DTH Responses

These experiments sought to determine if anti-LFA-1 and anti-ICAM mAb treatment altered the cell-mediated cytotoxicity and DTH responses of experimental mice. Heterotopic transplantation was specifically selected for these studies because placing a corneal graft onto a vascularized subdermal graft bed richly endowed with lymphatics maximizes sensitization of the host’s immune system. The mice were grafted on day 0, and mAb treatment followed the same protocol used in orthotopic transplantation experiments. Hosts were evaluated for DTH response on day 14. On day 16, the mice were killed and CTL assays were performed. Heterotopic corneal grafts (containing the peripheral Langerhans cell-rich limbus) induced potent CTL and DTH responses to donor alloantigens (Figs. 2 and 3). By contrast, hosts treated with anti-LFA-1, either alone or in combination with anti-ICAM-1, failed to develop either CTL or DTH responses that were any greater than naive controls. Although anti-ICAM-1 mAb alone strongly inhibited allospecific CTL and DTH responses, the suppressive effect was considerably less than anti-LFA-1 treatment (Figs. 2 and 3). Hosts treated with anti-LFA-1 experienced profound impairment of CTL and DTH responses to donor alloantigens and a marked reduction orthotopic corneal allograft rejection (Table 1). It was of interest, therefore, to determine that when graft rejection occurred in antibody-treated hosts, if it correlated with the acquisition of either CTL or DTH allospecific im-

![FIGURE 2. CTL responses in mice immunized heterotopically with corneal allografts and treated with anti-LFA-1 and anti-ICAM-1 mAbs. Antibody treatment was administered as described in Materials and Methods. Results of a typical 4-hour 51Cr-release assay in which spleen cells were pooled in each respective group 14 days after heterotopic grafting. Effector-to-target ratio was 50:1. There were five mice in each group.](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933406/)
mune responses. Similarly, it was important to determine if antibody-treated hosts bearing long-term clear corneal grafts retained depressed CTL and DTH responses.

Hosts previously treated with either anti-LFA-1 alone or with a combination of anti-LFA-1 and anti-ICAM-1 mAbs were categorized as rejectors or nonrejectors based on the fate of their orthotopic grafts by the 45th day after transplantation. Hosts that rejected their orthotopic grafts in spite of anti-LFA-1 antibody treatment or combined anti-LFA-1 and anti-ICAM-1 antibody treatment expressed potent cell-mediated cytotoxicity (Fig. 4). By contrast, hosts bearing clear corneal grafts (that is, nonrejectors) did not develop demonstrable cell-mediated cytotoxicity responses that were significantly greater than naive controls (Fig. 4). Interestingly, both groups of hosts failed to develop DTH responses (Fig. 5).

**Effect of In Vivo Administration of Anti-LFA-1 and Anti-ICAM-1 Antibodies on CD4+ and CD8+ T Cell Populations**

The efficacy of in vivo anti-LFA-1 and anti-ICAM-1 treatment was evaluated by FACS analysis of splenocytes from antibody-treated mice. The results summarized in Table 2 indicate that mAb treatment resulted in a great reduction of LFA-1 (35% to 17%) and ICAM-1 (15% to 5%) expression on splenocytes from allografted mice.

Because T lymphocytes express LFA-1 on the surface, it was of interest to determine if anti-LFA-1 mAb
Effect of Anti-LFA-1 on Corneal Graft Survival

TABLE 2. Effect of Combined Anti-LFA-1 and Anti-ICAM-1 Antibody Treatment on Lymphoid Cell Populations

<table>
<thead>
<tr>
<th>Cell Population</th>
<th>Normal Mice</th>
<th>Grafted Mice (Untreated)</th>
<th>Grafted mice (aLFA-1 + aICAM-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LFA-1*</td>
<td>60%</td>
<td>36%</td>
<td>17%</td>
</tr>
<tr>
<td>ICAM-1*</td>
<td>2%</td>
<td>15%</td>
<td>5%</td>
</tr>
<tr>
<td>CD4*</td>
<td>35%</td>
<td>58%</td>
<td>52%</td>
</tr>
<tr>
<td>CD8*</td>
<td>20%</td>
<td>39%</td>
<td>63%</td>
</tr>
</tbody>
</table>

Immunofluorescent staining of pooled spleen cell populations determined by FACS as described in Materials and Methods. There were five animals in each group.

Effect of Anti-LFA-1 on Corneal Graft Survival

The current study shows that in vivo treatment with mAb against LFA-1 produced a significant prolongation of corneal allograft survival and greatly reduced the incidence of rejection. A short period of in vivo treatment with anti-LFA-1 antibody, given prophylactically, prevented the induction of both CTL and DTH responses to donor alloantigens, even in hosts sensitized with highly immunogenic heterotopic corneal allografts. Most previous studies on the immunomodulating effect of anti-LFA-1 have focused on its inhibition of CTL induction. Our results, however, demonstrate that anti-LFA-1 mAb also prevented DTH responses. Thus, anti-LFA-1 inhibited the two major cellular immune effector mechanisms commonly associated with allograft rejection.

Effect of Anti-LFA-1 and Anti-ICAM-1 mAb Treatment in Inducing Tolerance

Despite the extraordinary success corneal grafts enjoy, a significant number fail because of immunologic rejection. Accordingly, allograft rejection is the leading cause of corneal transplant failure, especially in high-risk patients with previous graft rejection or whose corneas are vascularized. Thus, understanding the pathogenesis of corneal graft rejection and establishing novel regimens for prolonging graft survival are crucial.

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Efficacy of mAb Treatment on ‘High-Risk’ Hosts

One of the major challenges of therapeutic keratoplasty is preventing allograft rejection in “high-risk” patients who have become sensitized through the rejection of previous corneal grafts. Therefore, the efficacy of anti-LFA-1 and anti-ICAM-1 treatment in preventing corneal allograft rejection in sensitized hosts was examined. Accordingly, normal CB6F1 mice were sensitized with two heterotopic C3H corneal allografts. Fourteen days later, mice were treated with a combination of anti-LFA-1 and anti-ICAM-1 following the original protocol and were grafted orthotopically with C3H corneal allografts. Even though combined administration of anti-LFA-1 and anti-ICAM-1 mAbs reduced the rejection of primary orthotopic corneal allografts from 90% to 47% in first-time hosts, no beneficial effect was observed in presensitized hosts—100% of the orthotopic-challenge grafts underwent rejection (Fig. 1).

DISCUSSION

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Antibodies against LFA-1 have been used with different rates of success in preventing allograft rejection in various categories of organ transplantation. Fisher and coworkers reported that anti-LFA-1 (CD11a) mAb successfully prevented graft failure in children receiving HLA-mismatched bone marrow. Using the same mAb, Mauff et al were unsuccessful in reversing acute rejection of kidney transplants. However, the survival of cardiac and skin allografts in experimental animals was prolonged by administration of anti-LFA-1 mAb.

The success of anti-LFA-1 and anti-ICAM-1 mAb treatment in preventing allograft rejection is influenced by the host species (e.g., human versus rodent), as well as the isotype of mAb used (e.g., IgG1 vs IgG2). The results reported here indicate that the timing of antibody treatment also has a significant effect.

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on corneal allograft survival. Anti-LFA-1 mAb prevented graft rejection if given to nonimmunized hosts before corneal transplantation. However, anti-LFA-1 mAb was totally ineffectual in promoting graft acceptance in previously immunized hosts. These results suggest that anti-LFA-1 mAb acts to prevent the induction of alloimmune responses but does not interfere with the expression of effector mechanisms involved in orthotopic corneal allograft rejection.

It was puzzling that both anti-ICAM-1 and anti-LFA-1 significantly inhibited CTL and DTH responses, yet only anti-LFA-1 treatment impaired corneal allograft rejection. Because anti-ICAM-1 treatment did not reduce the incidence of corneal graft rejection, it is not surprising that combining both mAbs was not any more effective than anti-LFA-1 treatment alone in preventing rejection. A recent report using a rat model of keratoplasty indicated that combined treatment with anti-LFA-1 and anti-ICAM-1 prevented orthotopic corneal allograft rejection. Unfortunately, that study did not evaluate the effect of anti-LFA-1 or anti-ICAM-1 treatment alone.

LFA-1 influences a wide variety of immunologic events, including cell-mediated cytotoxicity, antigen presentation to T cells, T-helper and B-lymphocyte responses, antibody-dependent cytotoxicity by monocytes and granulocytes, and adherence of leukocytes to endothelial cells. It can interact with at least three counter ligands—ICAM-1, ICAM-2, and an undefined third ligand. The redundancy in counter ligands may explain the failure of anti-ICAM-1 mAb to inhibit corneal allograft rejection because the other counter ligands might have substituted for ICAM-1 in a variety of functions.

Although the mechanism by which anti-LFA-1 and anti-ICAM-1 mAbs exert immunosuppressive effects remains poorly understood, it is not a result of cytolytic deletion of relevant effector lymphocytes. Our results parallel previous studies demonstrating that anti-LFA-1 and anti-ICAM-1 treatment did not reduce the absolute numbers or percent of circulating CD4+ and CD8+ lymphocytes in experimental hosts yet significantly diminished the percent of lymphoid cells expressing ICAM-1 and LFA-1 cell adhesion molecules. Other investigators have suggested that the reduction in LFA-1+ and ICAM-1+ cells could be due to deletion of a unique effector cell population or downregulation in the expression of cell adhesion molecules. However, the precise mechanism remains a mystery.

Based on the limited data reported here, we favor the hypothesis that the primary effect of anti-LFA-1 mAb treatment was interferring with alloantigen presentation and not by disabling the migration and extravasation of sensitized effector T cells. Aggressive treatment with both anti-LFA-1 and anti-ICAM-1 failed to prevent corneal allograft rejection in primunized hosts. If the primary beneficial effect of anti-LFA-1 and anti-ICAM-1 mAb treatment is in preventing leukocyte homing, extravasation, and cytolysis of the corneal graft, then administration of antibodies to preimmune hosts should produce a beneficial effect on corneal allograft survival. However, aggressive treatment with both anti-LFA-1 and anti-ICAM-1 failed to prevent corneal allograft rejection in preimmunized hosts.

In conclusion, anti-LFA-1 mAb given either alone or in combination with anti-ICAM-1 mAb profoundly inhibited the induction of CTL and DTH responses by heterotopic corneal allografts. Moreover, prophylactic use of anti-LFA-1 mAb significantly enhanced orthotopic corneal allograft survival. This enhancement of corneal graft survival could not be improved by adding anti-ICAM-1 mAb or by a more aggressive treatment protocol. Moreover, neither anti-LFA-1 alone, nor the combination of both mAbs, could prevent the rejection of orthotopic corneal allografts in presensitized hosts. This finding implies that anti-LFA-1 acts to prevent the induction of alloimmune responses but does not affect the execution of alldestructive effector processes.

**Key Words**
keratoplasty, intercellular cell adhesion molecule, leukocyte function antigen, antibody, mouse

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**References**


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