Combined Blockade of VEGFR-3 and VLA-1 Markedly Promotes High-Risk Corneal Transplant Survival

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PURPOSE. High-risk corneal transplantation refers to grafting performed on inflamed and highly vascularized host beds. It represents a clinical dilemma because the rejection rate can be as high as 90%, irrespective of current treatment modalities. This study was conducted to investigate whether combined blockade of VEGFR-3 (vascular endothelial growth factor receptor-3) and VLA-1 (very late antigen-1) promotes high-risk transplant survival and how it correlates with corneal lymphangiogenesis and hemangiogenesis before and after transplantation.

METHODS. High-risk corneal transplantation was performed between normal C57BL/6 (donor) and inflamed BALB/c (recipient) mice. The recipients were randomized to receive intraperitoneal injections of VEGFR-3 and VLA-1-neutralizing antibodies or their controls twice a week for up to 8 weeks after transplantation. Corneal grafts were evaluated by ophthalmic slit-lamp biomicroscopy and analyzed by Kaplan-Meier survival curve. Additionally, whole-mount corneas before and after transplantation were examined by immunofluorescent microscopic assays, and the correlation between lymphatic or blood vessel distribution and transplant outcome was analyzed.

RESULTS. The combined blockade markedly promotes 90% survival of high-risk transplants. This strategy specifically modified host beds by selective inhibition of lymphangiogenesis but not hemangiogenesis. A strong correlation was also identified between high-risk transplant rejection and severe lymphatic invasion reaching the donor-graft border.

CONCLUSIONS. These novel findings not only provide a new and potentially powerful strategy to promote high-risk transplant survival, they also confirm a critical role of high-degree lymphangiogenesis in mediating high-risk transplant rejection. Results from this study may also shed new light on our understanding and management of other lymphatic- and immune-related diseases in general. (*Invest Ophthalmol Vis Sci*. 2011;52:6529–6535) DOI:10.1167/iovs.11-7454

Transplantation remains the last hope to restore the functions of a tissue or an organ to patients whose other treatments have failed or who are experiencing medical emergencies. This hope, however, is greatly jeopardized by im-

mune-mediated rejection, which is the primary reason for transplant failure.^{1–5} Among all solid organ or tissue transplantations, corneal transplantation is the most common and successful form; it enjoys a 2-year survival rate of 90% in patients with uninflamed and avascular (low-risk) graft beds. The rejection rate, however, dramatically increases and reaches as high as 90% when the grafting is performed on inflamed and highly vascularized (high-risk) corneas and the immune privilege of this site is compromised.^{1–3,5–7} To date, there is still little effective management of this high rejection situation. Unfortunately, many patients who are blind as a result of corneal diseases fall in this category after a traumatic, inflammatory, infectious, or chemical insult. It is, therefore, a field with an urgent demand for new therapeutic protocols.

Corneal transplantation also provides an ideal model for the study of allogeneic transplantation as it relates to vessel formation and regulation. This is largely because as the forefront tissue of the visual pathway, the normal adult cornea is both transparent and avascular. It is, therefore, both easy and straightforward to spot and assess conditions of the grafts and newly formed vessels in this tissue.^{2,8} Both lymphatic and blood vessels are involved in the immune reflex arc of transplantation, which mainly consists of the following components: the afferent pathway of lymphatic vessels through which antigens and antigen-presenting cells migrate to the draining lymph nodes, the lymph nodes where T cell priming occurs, and the efferent pathway of blood vessels through which the primed T cells are homed to the targeted grafts. In high-risk host corneas, cellular trafficking afforded by both lymphatic and blood vessel channels are greatly enhanced, which accelerates transplant rejection.^{1–3,5,9}

Compared with blood vessels that have been studied extensively in the past, lymphatic research has been neglected for centuries but has experienced exponential growth in recent years. This is largely because of the advancement of modern technologies and the discoveries of several lymphatic endothelial-specific molecules, including vascular endothelial growth factor receptor-3 (VEGFR-3), lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1), and Prox-1. The lymphatic network penetrates most tissues in the body, and its dysfunction has been found in a broad spectrum of disorders, such as cancer metastasis, inflammatory and immune diseases, tissue and organ (heart and kidney) transplant rejection, obesity, hypertension, and lymphedema.^{2,10–15} There are few effective treatments for lymphatic diseases, which defines another field with a great need for new therapeutic strategies.

Previous data from us and other researchers have demonstrated that VEGFR-3 mediates corneal lymphangiogenesis (LG; the development of new lymphatic vessels); its blockade suppresses donor-derived cell trafficking to draining lymph nodes and promotes transplant survival in normal or low-risk corneas.^{16–18} More recently, we also demonstrated that very late antigen-1 (VLA-1; also known as integrin $\alpha 1 \beta 1$) mediates corneal inflammatory LG in vivo and lymphatic endothelial cell functions in vitro.¹⁹ Its inhibition reduces macrophage, leuko-

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Supported in part by research grants from the National Institutes of Health, the Department of Defense, and the University of California at Berkeley (LC).

Submitted for publication February 25, 2011; revised May 24, 2011; accepted June 16, 2011.

Disclosure: **H. Zhang**, None; **S. Grimaldo**, None; **D. Yuen**, None; **L. Chen**, None

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cyte, and T cell infiltrations of low-risk corneal grafts, which also tend to survive better.²⁰ Although these preliminary data on VEGFR-3 and VLA-1 obtained from normal or low-risk transplantation studies are promising, it remains unknown whether it is possible to interfere with both pathways to promote high-risk transplant survival. Answers to this question are critical because although low-risk transplantation provides a relatively simple model with which to study transplantation immunity, investigation of high-risk transplantation is of more clinical importance. Indeed, most patients who do not respond to current treatment regimens of transplant rejection fall in the high-risk category. Because VEGFR-3 and VLA-1 exhibit distinctive yet overlapping functions involved in the afferent and efferent arms of the immune reflex arc, it is plausible to hypothesize that combined blockade of both pathways may promote high-risk transplant survival, which is investigated in the present study.

In this article, we present the first evidence showing that a combined blockade of VEGFR-3 and VLA-1 markedly promotes 90% survival of high-risk transplants compared with 20% of the control condition. Moreover, this strategy selectively suppresses LG in high-risk host beds, along grafting borders, and in donor buttons. A strong correlation between high-risk transplant rejection and severe lymphatic invasion across the donor-graft border is also revealed. These novel findings not only confirm a critical role of the lymphatic pathway in mediating high-risk transplant rejection, they also provide a new and potentially powerful strategy to combat high-risk transplant rejection and possibly other immune- or lymphatic-related diseases in the body.

METHODS

Animals

Six- to 8-week-old male C57BL/6 and BALB/c mice (Taconic Farms, Germantown, NY) were used in all experiments. All mice were treated according to ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and all protocols were approved by the Animal Care and Use Committee, University of California, Berkeley. Mice were anesthetized using a mixture of ketamine, xylazine, and acepromazine (50 mg, 10 mg, and 1 mg/kg body weight, respectively) for each surgical procedure.

Induction of Corneal Neovascularization for Creation of High-Risk Host Beds

High-risk host beds were created as previously described.^{9,21,22} Briefly, three interrupted intrastromal sutures (11-0 nylon, Sharp; Vanguard, Houston, TX) were placed in the central cornea of the BALB/c mouse to induce inflammatory lymphangiogenesis and hemangiogenesis. All sutures were removed 2 weeks later, and the neovascularized corneas served as high-risk host beds for corneal transplants.

Corneal Transplantation

Corneal transplantation was performed in inflamed high-risk host beds according to the standard protocol.^{17,20,23} Briefly, the central cornea of the donor of C57BL/6 mice was marked with a 2-mm diameter microcurette (Katena Products Inc., Denville, NJ) and excised with Vannas scissors (Storz Instruments Co, San Dimas, CA). The recipient graft bed was prepared by excising a circular 1.5-mm area in the central cornea. The donor button was placed onto the graft bed and secured with eight interrupted 11-0 nylon sutures (Sharp; Vanguard), followed by the application of antibiotic ointment.

In Vivo Assessment of Grafted Corneas

All grafted eyes were first examined 3 days after surgery, and corneal sutures were removed on day 7. Corneal grafts were observed in vivo

twice a week by ophthalmic slit-lamp biomicroscopy for 8 weeks and evaluated according to the standard grading scheme, as described previously.^{17,20} Briefly, the degree of opacification was graded between 0 (clear and compact graft) to 5+ (maximal corneal opacity with total obscuration of the anterior chamber). Grafts with an opacity score of 2+ or higher after 3 weeks or an opacity score of 3+ or higher at 2 weeks were regarded as rejected.

Pharmaceutical Interventions

After surgery, mice were randomized to receive intraperitoneal administrations of either neutralizing antibodies of VEGFR-3 (700 µg; kindly provided by ImClone Systems, Eli Lilly and Company [New York, NY]) and VLA-1 (200 µg; kindly provided by Covella Pharmaceuticals, Inc. [San Diego, CA] and Biogen Idec, Inc. [Cambridge, MA]) or their isotype controls twice a week on the day of suture placement and thereafter. As illustrated in Figure 1, to study the effect of antibody treatment on modification of the high-risk host beds, the treatment was given for 2 weeks until sutured corneas were sampled for immunofluorescent microscopic assays. The experiments were repeated twice with a total of 10 mice in each group. For transplantation studies, the treatment was given similarly up to 8 weeks after transplantation with a total of 10 mice in each group.

Immunofluorescence Microscopic Assay

The experiments were performed according to our standard protocol.^{19,21,22,24} Briefly, fresh corneas were excised, fixed, and incubated

FIGURE 1. Schematic diagram demonstrating experimental procedures. Normal BALB/c mice were subjected to corneal suture placement to induce high-risk host beds. Two weeks later, sutures were removed and corneal transplantations were performed between normal C57BL/6 (donor) and inflamed BALB/c (recipient) mice. Mice were observed and evaluated in vivo twice a week by ophthalmic slit-lamp microscopic examination up to 8 weeks after transplantation. Immunofluorescence microscopic assays were performed to investigate blood and lymphatic vessels 2 weeks after suture placement and 8 weeks after corneal transplantation, respectively.

with FITC-conjugated rat anti-mouse CD31 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) or rat anti-mouse CD31/PECAM-1 (BD PharMingen, San Diego, CA) followed by Alexa 488-conjugated donkey anti-rat IgG (Jackson ImmunoResearch, West Grove, PA). These samples were then stained with rabbit anti-mouse LYVE-1 (Abcam, Cambridge, MA), which was visualized by Cy3-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch). The samples were mounted with mounting medium (Vectashield; Vector Laboratories, Burlingame, CA), observed with a fluorescence microscope (Carl Zeiss, München-Hallbergmoos, Germany), and photographed with a digital camera system (AxioCam; Carl Zeiss). Vascular structures stained as CD31⁺LYVE-1⁻ were identified as blood vessels, whereas those stained as CD31⁺LYVE-1⁺ were defined as lymphatic vessels.

Vascular Quantification

Vessels in whole-mount corneas were graded and analyzed using the ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at <http://rsb.info.nih.gov/ij/index.html>), as described previously.^{19,21,22,24} Basically, the blood or lymphatic vessel area was normalized to the total corneal area to obtain a percentage coverage score for each sample. Additionally, vessels in three different zones of the cornea after transplantation were also graded, as described previously with some modifications.^{20,21,25,26} Briefly, the quantification of vessels in the host beds or donor buttons was based on two primary parameters. One was the circumferential extent of 12 areas around the clock. A score of 1 was given to each area if the vessels were present in the sector. The other was the centripetal growth of the longest vascular frond in each area. A grade between 0 (no growth) and 2 (at the grafting border for host bed vessel quantification or at the center of the cornea for donor button vessel quantification) was given to each area. Scores for each area were then summed to derive the final index (range, 0–24; maximal score, 24 = 2 × 12). Vessels along the donor-graft borders were quantified based on their presence around 12 clocks (range, 0–12).

Statistical Analysis

Results were expressed as the mean ± SEM, and Student's *t*-tests were performed to evaluate the statistical significance of the difference between the control and treatment groups. Corneal graft survival was assessed by Kaplan-Meier survival curves. The association between the ingrowth of lymphatic vessels into each graft and its survival outcome was analyzed by the χ^2 test. All statistical analysis was performed using statistical analysis software (Prism; GraphPad Software, Inc., La Jolla, CA). *P* < 0.05 was considered significant.

RESULTS

Combined Blockade of VEGFR-3 and VLA-1 Selectively Inhibits Inflammatory Lymphangiogenesis in High-Risk Host Beds

Because lymphatic and blood vessels constitute the afferent and efferent pathways of the immune reflex arc of transplantation immunity, we first set out to study the effect of the combined blockade of VEGFR-3 and VLA-1 on the formation of new blood and lymphatic vessels in inflamed corneas, which also served as recipient beds for high-risk transplantation. To approach this, a standard suture placement model was used in the mouse cornea to induce significant ingrowth of both vessel types. The effect of VEGFR-3 and VLA-1 blockade was analyzed by comparing vascularized conditions in neutralizing antibody treatment and control groups. As shown in Figure 2A (upper panels), though the combined blockade dramatically reduced inflammatory LG in the treatment group, this strategy demonstrated little effect on blood vessels (Fig. 2A, lower panels), which was also confirmed by *in vivo* slit-lamp microscopic examination (data not shown). Summarized data from repetitive experiments are presented in Figure 2B (*P* < 0.001).

FIGURE 2. Combined blockade of VEGFR-3 and VLA-1 selectively inhibited inflammatory lymphangiogenesis in the host beds induced by suture placement. (A) Representative whole-mount micrographs showing lymphangiogenesis after suture placement was significantly suppressed by the combined treatment. Blood vessels remained similar between the treatment and control groups. Green: CD31; red: LYVE-1. Original magnification, 100×. (B) Summarized data from repetitive experiments. BV, blood vessels; LV, lymphatic vessels. ***P < 0.001. n.s.: no significant difference.

Combined Blockade of VEGFR-3 and VLA-1 Markedly Improves the Transparency of High-Risk Grafts

We next performed high-risk transplantation on the inflamed and highly vascularized host beds and investigated the effect of the combined treatment on graft transparency, an important index for the outcomes of the transplants. Transplantation was performed between fully mismatched normal C57BL/6 (donor) and inflamed BALB/c (recipient) mice. All grafts in the treatment and control groups were evaluated *in vivo* twice a week by ophthalmic slit-lamp microscopy up to 8 weeks after transplantation. As demonstrated in Figures 3A and 3B, at all time points studied, the grafts in the treatment group enjoyed greater clarity than those in the control group. Summarized data are presented in Figure 3B ($P < 0.001$).

Combined Blockade of VEGFR-3 and VLA-1 Leads to Remarkable Survival of High-Risk Transplants

To further evaluate the effect of the combined treatment on high-risk transplant survival, we also followed up all grafts in the treatment and control groups and evaluated their survival rate twice a week up to 8 weeks after transplantation. It is known that the onset of high-risk corneal transplant rejection occurs around 2 to 3 weeks after surgery. As shown in Figure

3C with Kaplan-Meier survival curves, our results showed a remarkable promotion of transplant survival by this treatment. It was observed that graft rejection in the control group started earlier, from 2 weeks after transplantation compared with 4 weeks in the treatment group. Within 3 weeks after transplantation, 70% of the grafts were already rejected in the control group, whereas all those in the treatment group survived. By 4 weeks after transplantation, only 20% of the grafts survived in the control condition. Surprisingly, grafts in the treatment condition enjoyed a high survival rate of 90%, which remained until the end of the 8-week study ($P < 0.005$). This treatment, therefore, suppressed both the onset and the scale of the rejection.

Combined Blockade of VEGFR-3 and VLA-1 Suppresses Lymphangiogenesis in Host Beds, along Donor-Graft Borders, and into Donor Buttons

We next sought to examine whether the combined treatment affected the ingrowth of lymphatic or blood vessels into grafted corneas after high-risk transplantation. To approach this, whole-mount intact corneas (including both host beds and donor buttons) from the treatment and control groups were collected by the end of the 8-week transplantation study and subjected to immunofluorescence microscopic assays using both anti-CD31 and anti-LYVE-1 antibodies. The effect of the combined treatment on lymphatic and blood vessels was analyzed in whole-mount corneas as well as three individual zones defined in grafted corneas. These included host beds, donor-graft borders and donor buttons, respectively (zone a-c, Fig. 4C). As shown in Figures 4A and 4B for whole corneal studies, lymphatic vessels in the treated corneas were significantly reduced ($P < 0.001$), whereas no significant effect was observed for blood vessels. Further analysis on the three different zones in the grafted corneas also revealed that while lymphatic vessels in the host beds were greatly suppressed by the treatment, those in the graft buttons or along the donor-graft borders were completely abolished (Fig. 4E; $P < 0.001$). No significant differences were observed in all three areas for blood vessels (Fig. 4D).

Lymphangiogenesis Crossing the Donor-Graft Border Correlates with High-Risk Transplant Rejection

Finally, we also examined each cornea in the treatment and control groups and compared the transplant outcome with the degree of lymphatic or blood vessel invasion in each sample. Interestingly, it was found that corneal grafts with few (Fig. 5A) or no lymphatic vessels (Fig. 5C) were more likely to survive in both treatment and control groups. In clear contrast, those grafts with significant lymphatic invasion were rejected (Fig. 5B). Moreover, after performing an association analysis between the degree of LG and graft rejection, we found that the grafts with severe LG reaching the donor-graft borders were rejected while those not affected by this high degree of LG survived. A strong correlation between these two parameters was, therefore, identified for high-risk transplantation, as summarized in Figure 5D ($P < 0.005$).

DISCUSSION

In this study, we provide the first evidence showing a novel strategy to promote high-risk transplant survival. At least three important conclusions can be drawn from our data. First, a combined blockade of VEGFR-3 and VLA-1 is highly effective in suppressing LG in the inflamed host beds before and after transplantation. This treatment acts to normalize the inflamed

FIGURE 3. Combined blockade of VEGFR-3 and VLA-1 blockade markedly promoted the outcomes of high-risk grafts. (A) Representative *in vivo* slit-lamp pictures showing rejected and survived corneal grafts in control and treatment groups 8 weeks after transplantation, respectively. (B) Summarized data showing grafts in the treatment group have lower opacity scores with greater clarity at all time points studied. *** $P < 0.001$. (C) Kaplan-Meier survival curves showing significantly higher rates of graft survival in the treatment group. ** $P < 0.005$.

FIGURE 4. Combined blockade of VEGFR-3 and VLA-1 selectively inhibited lymphangiogenesis in the corneas after high-risk transplantation. **(A)** Representative whole-mount micrographs showing lymphangiogenesis in grafted corneas was significantly suppressed in the treatment group. Blood vessels were not affected. *Green*: CD31; *red*: LYVE-1. Original magnification, 50 $\times$. **(B)** Summarized data for whole cornea assays. *** $P < 0.001$. **(C)** Schematic diagram demonstrating three individual zones analyzed on corneal vessels after transplantation. *Gray*: zone a, host bed; *blue*: zone b, donor button; *dotted line, arrow*: zone c, donor-graft border. **(D)** Summarized data showing no significant difference on blood vessels in all three zones analyzed between the treatment and control groups. **(E)** Summarized data showing that while lymphangiogenesis was significantly suppressed by the treatment in host beds, it was completely eliminated along the grafting borders and in donor buttons. BV, blood vessels; LV, lymphatic vessels; n.s., no significant difference. *** $P < 0.001$.

tissues and to revert them to a lymphatic-low status. Second, this strategy remarkably promotes transplant survival in the inflamed tissues, which are still endowed with a large amount of blood vessels, indicating that lymphatic but not blood vessels primarily mediate high-risk lymphatic rejection. Given that blood vessels are important for many other functions essential for transplant survival, such as nutrient supply and wound healing, this novel strategy has the great advantage of selectively inhibiting the unfavorable immune responses while sparing other important functions for transplant survival. Third, a high degree of LG crossing the grafting border contributes to high-risk graft rejection. Reducing LG to a lower level, therefore, should be considered in our future efforts to develop new therapies for high-risk transplant rejection.

Our data showing that high-risk transplant survival is strongly associated with LG across the grafting border is new but consistent with previous studies showing that the lymphatic pathway is essential for mediating corneal transplant rejection. It has been demonstrated that surgical severing of the lymphatic pathway by the removal of draining lymph nodes promoted 100% and 90% of graft survival in low- and high-risk settings, respectively.^{23,27} However, surgical lymphadenectomy for promoting transplant survival is not practical in human patients. It is, therefore, critical to identify molecular

factors involved in this pathway. This present study thereby bears more clinical significance and may provide an alternative method of molecular lymphadenectomy¹⁷ to promote high-risk transplant survival.

To our knowledge, there have been no studies to date showing the dramatic survival of high-risk transplants as presented in this report. A few molecular pathways have been investigated in the past for high-risk transplant survival, including proinflammatory cytokine IL-1, the costimulatory CD40L (CD154)-CD40, VEGF-A, and VEGFR-3.^{11,28–30} Although the graft survival rate is improved in all these cases, we have only seen results approaching what we observed in this study with systemic anti-CD40L treatment, in both cases achieving 90% graft survival. However, the development of the anti-CD40L strategy in the clinic was impeded by serious thrombotic adverse effects in human patients with systemic autoimmune diseases.³¹ In this study, we are able to achieve marked graft survival without any apparent toxicity effects because both antibodies were used at low doses within their safe working ranges. Anti-VEGF-A and anti-VEGFR-3 treatments promote only 23% and 50% graft survival, respectively,^{11,30} both of which are significantly lower than what we achieved in this study. Besides graft survival, we demonstrated a strong correlation between high-degree LG crossing the donor-graft border

FIGURE 5. Lymphangiogenesis reaching the grafting border was highly associated with high-risk transplant rejection. (A–C) Representative whole-mount micrographs showing a survived graft with few lymphatic vessels in the control group (A), a rejected graft with significant lymphatic ingrowth across the donor-recipient border in the control group (B), and a survived graft without lymphatic invasion in the treatment group (C). Red: LYVE-1. Original magnification, 25 $\times$. (D) Summarized data showing significant association between high-degree lymphangiogenesis and transplant rejection. LV, lymphatic vessels. ** $P < 0.005$.

and transplant rejection by observing each individual grafted cornea, which was not reported in previous studies.

The surprisingly high survival rate with the combined treatment may be explained by the fact that in addition to LG, other innate and adaptive aspects of transplantation immunity are regulated. For example, it is known from our low-risk study that neutrophil, macrophage, and T-cell infiltrations are suppressed by VLA-1 inhibition,²⁰ and the trafficking of antigen-presenting cells to draining lymph nodes is inhibited by VEGFR-3 blockade.¹⁷ A synergistic interplay between the VLA-1 and VEGFR-3 pathways may also exist and may contribute to the high success rate. VEGFR-3 is a receptor tyrosine kinase expressed on lymphatic endothelium, and integrins are ubiquitous heterodimeric proteins important for cell-cell and cell-extracellular matrix interactions.^{32,33} It has been demonstrated that VEGFR-3 selectively associates with integrin $\beta 1$ and that their synergistic interactions modulate the functions of lymphatic endothelial cells.^{34,35} It is, therefore, possible that a combined blockade of VEGFR-3 and VLA-1 interferes with the synergy between these two pathways and promotes transplant survival, which warrants further investigation.

Studies on the mechanisms of corneal LG and high-risk transplant rejection are important because LG accompanies many corneal diseases after inflammatory, infectious, chemical, or traumatic damages. The inflamed and lymphatic-rich corneas become hostile to transplants, irrespective of current treatment regimens.^{2,3,5} Because of the poor prognosis, many patients are not even considered good candidates for transplantation surgery and have to give up their hope for vision restoration. The pharmacotherapy of transplant rejection has changed little in the past decades despite the fact that corticosteroids are of limited efficacy and are fraught with serious side effects, such as glaucoma, cataracts, and opportunistic infections. It is anticipated that this study may offer new insights into high-risk transplantation immunity and may provide new immunotherapies to combat high-risk transplant rejection.

Moreover, this study bears broader implications beyond the treatment of ocular diseases alone. As mentioned earlier, numerous diseases are associated with lymphatic and immune

dysfunction in the body, which can be disfiguring, disabling, and even life threatening. During past few years, LG has emerged as a focus of research to reduce cancer metastasis and to promote major solid organ transplant survival.^{10,13,14} It is anticipated that beyond its contributions to eye diseases, this research will shed some light on the development of new therapeutic strategies to treat other lymphatic- and immune-related diseases in general.

Acknowledgments

The authors thank Statistical Consulting Service at University of California Berkeley for assistance with data analysis; Covella Pharmaceuticals, Inc. and Biogen Idec, Inc., for providing VLA-1 blocking antibodies; and ImClone Systems, Eli Lilly and Company, for providing VEGFR-3 blocking antibodies.

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