**Very Late Antigen 1 Blockade Markedly Promotes Survival of Corneal Allografts**

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**Objective:** To investigate the role of very late antigen 1 (VLA-1) (also known as integrin receptor α5β1) in corneal transplantation inflammation and allograft survival.

**Methods:** Cell infiltration and vasculogenesis (both angiogenesis and lymphangiogenesis) associated with allogeneic corneal transplantation were assessed in VLA-1–deficient conditions and controls by immunofluorescent microscopic studies. Corneal allograft survival was also assessed after anti–VLA-1 antibody treatment and in VLA-1 knockout recipient mice.

**Results:** Anti–VLA-1 antibody treatment leads to a profound reduction in the granulocytic, monocytic, and T-cell infiltration after corneal transplantation. In addition, corneal angiogenesis and lymphangiogenesis were both significantly suppressed in VLA-1 knockout mice. Remarkably, universal graft survival was observed in both anti–VLA-1 antibody treatment and knockout mice.

**Conclusions:** Very late antigen 1 blockade markedly reduces inflammation and inflammation-induced tissue responses, including vasculogenic responses, associated with corneal transplantation and promotes allograft survival.

**Clinical Relevance:** These studies offer insights into important integrin-mediated mechanisms of corneal transplant–related inflammation and provide possible new integrin-based immunotherapies for transplant rejection.

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**INTEGRINS ARE A DIVERSE FAMILY OF**

heterodimeric cell surface transmembrane glycoproteins that mediate cell–cell and cell–matrix interactions. Very late antigen 1 (VLA-1), integrin α5β1, is primarily a receptor for collagens and laminins. It has been shown that deletion of integrin α5 in mouse permits normal development but gives rise to a specific deficit in cell adhesion and attenuated delayed-type hypersensitivity. Furthermore, VLA-1 blockade ameliorates certain immunoinflammatory diseases such as arthritis. However, the roles of VLA-1 in organ transplant survival have never been investigated before, which is the major goal of this study.

Though corneal transplantation is by far the most common form of solid tissue transplantation in humans, its pharmacotherapy has changed little over the past several decades, though it is well known that the mainstay regimen with corticosteroids is only variably effective and associated potentially with serious adverse effects such as glaucoma, cataracts, and opportunistic infections. It is therefore important to explore more effective strategies to improve corneal transplant survival. Lymphatic and blood vessels play important roles in transplant immunity: lymphatics, allowing for antigen-presenting cell migration to lymph nodes and blood vessels, facilitating immune cell targeting of the graft. Indeed, previous data from our laboratory have shown that surgical excision of the local draining lymph nodes leads to indefinite and universal graft acceptance without any form of immunosuppression. However, surgical lymphadenectomy to promote graft survival is not practical. It is hence critical to investigate the molecular mechanisms underlying this pathway. Unlike angiogenesis, which has been extensively studied, the molecular regulation of lymphangiogenesis has historically been neglected for decades until recently when several lymphatic-specific markers were discovered. We have recently shown that blockade of vascular endothelial growth factor receptor 3 (VEGFR-3), a lymphatic molecule, greatly suppresses corneal transplant rejection. Though it has been shown previously that angiogenesis is suppressed in α5-deficient mice, the issue of lymphangiogenesis has never been addressed directly in those studies.
MICE AND ANTIBODIES

Very late antigen 1 knockout BLAB/c mice were generated as described previously10 and kindly provided by Biogen Idec (Cambridge, Mass). Seven- to 10-week-old, male, wild-type BALB/c or C57BL6 mice (Taconic Farms, Germantown, NY, or from our own breeding facility) were used in all other experiments. All protocols were approved by the Schepens Eye Research Institute Animal Care and Use Committee, and all animals were treated according to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. Mice were anesthetized using a mixture of ketamine hydrochloride and xylazine (120 and 20 mg per kilogram of body weight, respectively) for each surgical procedure. The following antibodies were used for this study: mouse Gr1–fluorescein isothiocyanate conjugated (FITC), mouse Mac-1–FITC, mouse CD31–FITC (Santa Cruz Biotechnology, Santa Cruz, Calif), purified antimouse LYVE-1 (lymphatic vessel endothelial hyaluronan receptor 1) (a kind gift of David Jackson, PhD, Weatherall Institute of Molecular Medicine, United Kingdom25), purified antimouse CD3, rat antimouse CD16/32, Rhodamine-conjugated donkey antirabbit IgG (Santa Cruz Biotechnology), Cy3-conjugated antihamster IgG (Jackson ImmunoResearch Laboratories, Inc, West Grove, Pa). Isotype controls included rat IgG2a–FITC, hamster IgG1, and rabbit serum. Purified VLA-1–blocking antibody (Ha 31/8) and the isotype control antibody (Ha 4/8) were kindly supplied by Biogen Idec. All the other antibodies (except where noted) and isotype-matched controls were purchased from BD Pharmingen, San Diego, Calif. For each antibody staining study on whole-mount tissues, 3 to 5 samples were chased from BD Pharmingen, San Diego, Calif. and examined by an epifluorescence microscope (Eclipse E800; Nikon, Japan). Digital pictures were taken using the Spot Image Analysis system, and vascular structures stained as CD31–LYVE-1+ were identified as blood vessels while those stained as CD31+LYVE-1 were defined as lymph vessels.16 Angiogenesis and lymphangiogenesis were graded according to our standard protocol as described previously, with some modifications.16,17 Briefly, the quantification was based on 2 primary parameters: (1) the circumferential extent (12 areas around the clock) of the vasculogenesis. A score of 1 was given to each area if the vasculogenesis was present in the sector. (2) the centripetal growth of the longest vascular front in each area. A grade between 0 (no growth) and 2 (vasculogenesis to the donor-graft border) was given to each area. Scores for each area were then summed to derive the vasculogenic index (range, 0-24). The mean difference between vasculogenesis scores was analyzed by the Mann-Whitney U test. For cross-section studies, the Gr-111, Mac-111, or CD3111 cells were counted for each section, covering the entire thickness and span of the corneal tissues, and correlation analysis between the number of Gr-11, Mac-11, and CD31 cells per section was performed with a t test. P<.05 was considered significant.

IMMUNOHISTOCHEMICAL STUDY AND EPIFLUORESCENCE MICROSCOPY

Briefly, eyeballs or whole-mount corneas were excised from mice. For cell infiltrate studies, 8-µm frozen sections were fixed in acetone for immunofluorescent staining as described previously.15 To block nonspecific staining, sections were blocked with 2% bovine serum albumin and anti-FcR monoclonal antibody (CD16/CD32) for 30 minutes before they were stained with primary or control antibodies for 2 hours. Thereafter, the sections were incubated with secondary antibodies for 1 hour. For vasculogenesis studies, the whole-mount flat corneas of VLA-1 knockout BALB/c mice were sampled 7 days posttransplantation and stained overnight with FITC anti–CD31 (PECAM-1) antibody and then anti–LYVE-1 (to specifically detect lymphatics) antibody for 1 hour. Finally, sections were covered with mounting medium (Vector, Burlingame, Calif) and examined by epifluorescence microscopy (Eclipse E800; Nikon, Japan). Digital pictures were taken using the Spot Image Analysis system, and vascular structures stained as CD31+LYVE-1+ were identified as blood vessels while those stained as CD31+LYVE-1 were defined as lymph vessels.16 Angiogenesis and lymphangiogenesis were graded according to our standard protocol as described previously, with some modifications.16,17 Briefly, the quantification was based on 2 primary parameters: (1) the circumferential extent (12 areas around the clock) of the vasculogenesis. A score of 1 was given to each area if the vasculogenesis was present in the sector. (2) the centripetal growth of the longest vascular front in each area. A grade between 0 (no growth) and 2 (vasculogenesis to the donor-graft border) was given to each area. Scores for each area were then summed to derive the vasculogenic index (range, 0-24). The mean difference between vasculogenesis scores was analyzed by the Mann-Whitney U test. For cross-section studies, the Gr-111, Mac-111, or CD3111 cells were counted for each section, covering the entire thickness and span of the corneal tissues, and correlation analysis between the number of Gr-11, Mac-11, and CD31 cells per section was performed with a t test. P<.05 was considered significant.

RESULTS

VLA-1 IS EXPRESSED ON GRAFTED CORNEAS

We first set to confirm the expression of VLA-1 on grafted corneas. As shown in Figure 1, VLA-1 staining was detected in the grafted corneal stroma (Figure 1A) while the isotype control sample staining was negative (Figure 1B).

VLA-1 BLOCKADE DOWN-REGULATES CELL INFILTRATION AFTER CORNEAL TRANSPLANTATION

We next investigated whether VLA-1 plays a role in the cell infiltration associated with corneal transplantation.
by studying the effect of VLA-1 blockade on both inflammatory-cell (Gr-1$^+$ neutrophils and Mac-1$^+$ monocytes/macrophages) and T-cell (CD3$^+$) infiltration into corneal grafts at various points. As seen in Figure 2, compared with the isotype control treatment groups, significant suppression of cell infiltration was observed with all the cell types studied in VLA-1 blockade groups at day 14 and day 28 ($P<.05$), before the onset of murine corneal allograft rejection (which typically occurs at 4 weeks after transplantation).

**VASCULOGENESIS IS SUPPRESSED IN THE CORNEAL GRAFTS OF VLA-1–DEFICIENT MICE**

Because growth of blood and lymphatic vessels into the normally avascular corneal bed is a critical facet of the local inflammatory response to grafts, and a major risk factor for subsequent graft rejection,$^{7,8}$ we subsequently examined the effect of VLA-1 deficiency in corneal transplantation–associated angiogenesis and lymphangiogenesis. As demonstrated in Figure 3, 7 days after transplantation, both blood (CD31$^+$LYVE-1$^-$) and lymph vessels (CD31$^+$LYVE-1$^+$) were significantly decreased in the VLA-1 knockout recipients (Figure 3B, D, and F) compared with wild-type controls (Figure 3A, C, and E) (results are summarized in Figure 3G) ($P<.01$).

**CORNEAL GRAFT SURVIVAL IS MARKEDLY ENHANCED WITH VLA-1 BLOCKADE OR IN VLA-1–DEFICIENT MICE**

The earlier data implicate VLA-1 in corneal transplantation–associated cell infiltration, as well as angiogenesis and lymphangiogenesis, which are all critical factors in corneal graft rejection. We then tested the central hypothesis that VLA-1 blockade improves corneal allograft survival by assessing the transplant survival in both anti–VLA-1 antibody treatment and in VLA-1 knockout recipient mice. Results from these studies are presented in Figure 4, summarized by Kaplan-Meier survival curves. Remarkably, universal graft survival was observed in both groups compared with their correspond-
Previously studied the effect of blockade of a number of molecular pathways in corneal transplantation immunity, including: (1) proinflammatory cytokines IL-1 and tumor necrosis factor α; (2) intercellular adhesion molecule 1; (3) the costimulatory CD40L(CD154)-CD40 pathway; and (4) the vascular endothelial growth factors (VEGFs) VEGF-A and VEGFR-3. Tho29

Figure 3. Representative whole-mount micrographs showing that both angiogenesis and lymphangiogenesis are suppressed in the corneal grafts in very late antigen 1–deficient mice. A, CD31 in controls. B, CD31 in knockout mice. C, LYVE-1 (lymphatic vessel endothelial hyaluronan receptor 1) in controls. D, LYVE-1 in knockout mice. E, Merged CD31 and LYVE-1 in controls. F, Merged CD31 and LYVE-1 in knockout mice. G, Summary of results. Error bars represent SEM. *P<.01 (original magnification ×200).
molecule interacts with VEGF-C and VEGF-D, the li-
been made for the role of integrins in lymphangiogenesis.

only seen results approaching what we see with block-
ade of VLA-1 with systemic anti-CD40L treatment, in both
these cases achieving universal graft survival. The de-
velopment of the anti-CD40L strategy in the clinic was
impeded, however, by serious thrombotic adverse ef-
facts in human subjects receiving the anti-CD40L treat-
ment for systemic autoimmune diseases.

The surprisingly high survival rate in VLA-1–blockade
or VLA-1–deficient conditions may be explained by the fact
that this molecular pathway is involved in both innate and
adaptive aspects of corneal transplantation immunity, since
both innate (neutrophil and macrophage) and T-cell in-
filtrations are suppressed. Our finding that corneal trans-
plantation–related angiogenesis is significantly sup-
pressed in VLA-1 knockout beds is consistent with previous
reports on suppression of tumor-associated angiogenesis
in α1-null mice and in VEGF-induced angiogenesis when
α1-blocking antibodies are administered. However, these
studies shed no light on lymphangiogenesis, a critical fac-
tor in tumor metastasis and the generation of immune re-
sponses in the cornea. In fact, only scarce references have
been made for the role of integrins in lymphangiogenesis.
A recent in vitro study on integrin αβ showed that this
molecule interacts with VEGF-C and VEGF-D, the li-
gands for the lymphangiogenic receptor VEGFR-3. However,
no in vivo studies have been reported regarding the
molecule. Moreover, knockout mice die shortly after
birth because of severe lymphatic deficiency. Indeed,
knockout mice cannot survive most of the specific ly-
phatic factors (such as VEGFR-3, Prox-1, VEGF-C, and
podoplanin). The luxury of viable VLA-1 knockout mice,
combined with the unique feature of the alymphatic sta-
tus of normal cornea, enables us to present herein the first
report, to our knowledge, on the in vivo role of VLA-1 on
transplantation-related lymphangiogenesis. Additionally,
our data that VLA-1 treatments lead to both the decrease
in Mac-1–cell infiltration and the suppression of cor-
neal lymphangiogenesis further support the recently re-
ported role for macrophages in lymphangiogenesis.

The lymphatic system penetrates most tissues in the
body, and its dysfunctions are involved in a diverse ar-
ray of diseases including lymphedema (primary to sec-
tory to cancer surgeries or radiation therapy), de-
layed wound healing, diabetes mellitus, and cancer cell
metastasis, which can be disabling, disfiguring, and even
life threatening. To date, there are no effective treat-
ments for them. It is anticipated that this study, beyond
its contributions to corneal transplant immunity, will also
shed light on the development of new therapeutic strat-
egies for other disorders associated with lymphangi-
genesis and inflammation.

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Figure 4. Kaplan-Meier survival curves showing the role of very late anti-
gen 1 (VLA-1) in corneal graft survival. Universal graft survival was ob-
served in both VLA-1 knockout (A) and VLA-1–neutralizing antibody treatment
groups (B) (n=10 for each experimental group). *P<.05.

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**Ophthalmological Numismatics**

Hermenegildo Arruga (1886-1972) of Barcelona, Spain, was an internationally renowned ophthalmologist and one of the first to practice and improve on Gonin’s technique of retinal detachment repair. In 1950, he was awarded the Gonin Medal, which is the highest award of ophthalmology.

In 1970, a commemorative medal of Arruga was struck by Calico of Barcelona, Spain. The obverse depicts the bust of Arruga facing ahead and slightly left. The reverse depicts rays of light in the sky coming through clouds; “... Et Facta Est Lux,” or translated, “and there was light,” from Genesis, is the reverse inscription.

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