Integrin Alpha 9 Blockade Suppresses Lymphatic Valve Formation and Promotes Transplant Survival

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Purpose. The lymphatic pathway mediates transplant rejection. We recently reported that lymphatic vessels develop luminal valves in the cornea during lymphangiogenesis, and these valves express integrin alpha 9 (Itga-9) and play a critical role in directing lymph flow. In this study, we used an allogeneic corneal transplantation model to investigate whether Itga-9 blockade could suppress valvulogenesis after transplantation, and how this effect would influence the outcomes of the transplants.

Methods. Orthotopic corneal transplantation was performed between fully mismatched C57BL/6 (donor) and BALB/c (recipient) mice. The recipients were randomized to receive subconjunctival injections of either Itga-9 blocking antibody or isotype control twice a week for 8 weeks. Corneal grafts were assessed in vivo by ophthalmic slit-lamp biomicroscopy and analyzed using Kaplan-Meier survival curves. Additionally, whole-mount full-thickness corneas were evaluated ex vivo by immunofluorescent microscopy on both lymphatic vessels and valves.

Results. Anti–Itga-9 treatment suppressed lymphatic valvulogenesis after transplantation. Our treatment did not affect lymphatic vessel formation or their nasal polarized distribution in the cornea. More importantly, Itga-9 blockade led to a significant promotion of graft survival.

Conclusions. Lymphatic valvulogenesis is critically involved in transplant rejection. Itga-9 targeting may offer a new and effective strategy to interfere with the immune responses and promote graft survival.

Keywords: lymphatic valve, lymphangiogenesis, transplantation, integrin alpha 9

The lymphatic vasculature system has essential functions in maintaining body fluid homeostasis, dietary fat absorption, and immune surveillance. Dysfunction of the lymphatic system has been found in a wide array of diseases and disorders from cancer metastasis to inflammation and transplant rejection.[1–3] In transplantation, graft failure is mainly due to rejection, but the existing treatments are of limited efficacy. Studies have shown that transplantation immunity can be modulated by a molecular blockade of the lymphatic pathway,[4–6] and lymphatic vessels have emerged as key modulators for the development of new therapeutic strategies.

Although most of the studies have been focused on the regulation of the formation of the lymphatic vessels, or lymphangiogenesis (LG), we have recently revealed that lymphatic vessels develop luminal valves as LG progresses, and these valves play a crucial role in guiding the flow of the lymph inside the vessels, which contains immune cells and antigens for immune responses.[7–9] It is yet to be determined whether an intervention of the formation of these lymphatic valves, or valvulogenesis (VG), can modulate transplant survival, which is a focus of this study.

Integrin alpha 9 (Itga-9) belongs to the integrin family that mediates cell-cell and cell-matrix interactions.[10] Previously, we reported that this molecule is highly expressed on newly formed lymphatic valves in the cornea,[7–9] and its gene knockdown can inhibit the functions of human dermal lymphatic endothelial cells in vitro, such as proliferation, adhesion, migration, and tube formation. We demonstrated

with a suture placement model that Itga-9 blockade can suppress inflammatory VG in a brief 2-week study.[11] These preliminary results indicate a perfect opportunity for us to elucidate (1) whether Itga-9 blockade can be used as a new strategy to interfere with VG and/or LG induced by transplantation, which is a much more complicated procedure than inflammation, as transplantation triggers immune responses against foreign antigens and a greater degree of VG and LG; (2) whether VG plays a critical role in transplant rejection, which to date, there has been no report on this aspect; and (3) whether Itga-9 blockade can be used to improve the outcomes of transplants. The current regimens of corticosteroids are of limited efficacy and also associated with many side effects, such as opportunistic infection, glaucoma, and cataract. Lymphatic-specific targeting may offer a more precise approach to promote graft survival. Therefore, results from this study may not only offer new insights into transplantation immunity but also provide a novel strategy to treat transplant rejection, and possibly other lymphatic- and immune-related diseases.

Methods

Animals

Six- to 8-week-old male BALB/c and C57BL/6 mice (Taconic Farms, Germantown, NY, USA) were used in the experiments, and mice were anesthetized using a mixture of ketamine, xylazine, and acepromazine (50 mg, 10 mg, and 1 mg/kg body
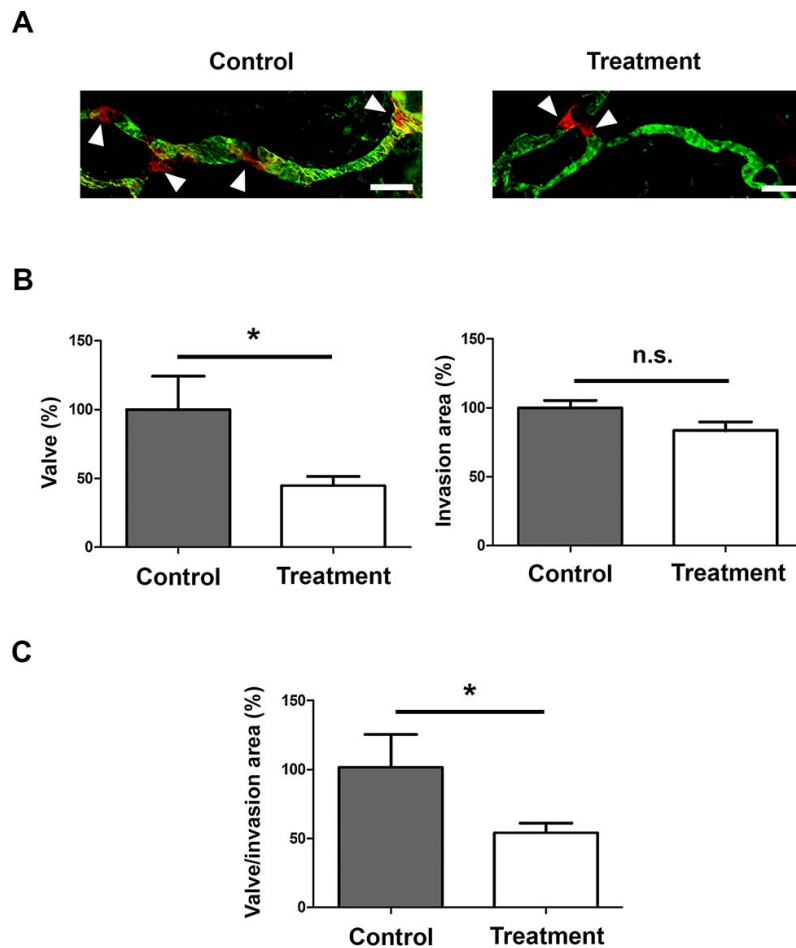
FIGURE 1. Lymphatic VG was suppressed by Itga-9 blockade after corneal transplantation. (**A**) Representative whole-mount immunostaining images demonstrating significantly fewer valves in the Itga-9 blocking antibody–treated cornea in comparison with isotype control–treated cornea. *Red*: Itga-9; *Green*: LYVE-1. *Scale bars*: 100 µm. (**B**) Comparative quantification on lymphatic valves and lymphatic vessel invasion area in control and treatment conditions. Anti–Itga-9 treatment only reduced valve formation. The experiment was repeated twice with seven mice in control and eight mice in treatment group. *$P < 0.05$; n.s., not significant. (**C**) Comparative quantification showing significant lower ratio of valves to lymphatic invasion area in response to anti–Itga-9 treatment. The experiment was repeated twice with seven mice in control and eight mice in treatment group. *$P < 0.05$.

weight, respectively) for each surgical procedure. All mice were treated according to the 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.

Corneal Transplantation

Orthotopic corneal transplantation was performed between fully mismatched C57BL/6 (donors) and BALB/c (recipients), as reported previously.[5,8] Basically, donor central cornea was marked with a 2-mm-diameter microcurette (Katena Products, Inc., Denville, NJ, USA) and excised with Vannas scissors (Storz Instruments Co., San Dimas, CA, USA). The recipient graft bed was similarly prepared with a 1.5-mm-diameter microcurette and the donor button was secured in recipient bed with eight interrupted 11-0 nylon sutures (AROSurgical, Newport Beach, CA, USA). Antibiotic ointment was applied at the end of the surgery.

Pharmaceutical Intervention

The recipient mice were randomized to receive subconjunctival injections of either hamster anti-mouse Itga-9 antibody (6.4 µg; kindly provided by Toshimitsu Uede, MD, PhD, Hokkaido University, Hokkaido, Japan) or its isotype control

hamster IgG (Jackson ImmunoResearch, West Grove, PA, USA), as reported previously.[11] Subconjunctival injection was performed twice a week on the day of transplantation and thereafter up to 8 weeks after the surgery.

In Vivo Assessment of Grafted Corneas

As described previously,[5] after the transplantation surgery, all eyes were first examined on day 3 and corneal sutures were removed on day 7. Grafts were evaluated by ophthalmic slit-lamp biomicroscopy twice a week for 8 weeks according to the standard scheme. Basically, the degree of graft opacification was graded between 0 (clear and compact graft) to 5+ (maximal 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.

Corneal Immunofluorescent Microscopy

The experiment was performed as described previously.[7–9] Briefly, whole-mount full-thickness corneas were harvested at 8 weeks after transplantation and fixed in acetone for immunofluorescent staining. Samples were sequentially incubated with purified rabbit anti-mouse LYVE-1 (Abcam, Cambridge, MA, USA) antibody and goat anti-mouse Itga-9 antibody (R&D

Systems, Minneapolis, MN, USA), which were visualized by FITC-conjugated donkey anti-rabbit and Cy3-conjugated donkey anti-goat secondary antibodies (Jackson ImmunoResearch Laboratories), respectively. Samples were covered with Vector Shield mounting medium (Vector Laboratories, Burlingame, CA, USA) and examined by an AxioImager M1 epifluorescence deconvolution microscope with AxioVision 4.8 software (Carl Zeiss AG, Göttingen, Germany).

Lymphatic Vessel and Valve Quantification

The analysis was performed as reported previously.[9,11–14] Briefly, for LG evaluation, LYVE-1^{+} vascular structures were analyzed by ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA).[9,12,13] The lymphatic invasion area was normalized to the total corneal area to obtain a percentage coverage score for each sample. The total corneal area was measured by outlining the innermost lymphatic vessels of the limbal arcade, and lymphatic invasion area was determined by tracing out the contours of the LYVE-1^{+} lymphatic network inside the cornea. Additionally, the cornea was divided into four equal quadrants in reference to the vertical midline passing through the 6- and 12-o'clock positions, and the nasal and temporal quadrants were used for analysis of polarized lymphatic vessel distribution for each sample.[9] Luminal valves also were evaluated and focal Itga-9^{+}/LYVE-1^{−} areas running along the length of the LYVE-1^{+} vessels were identified as valves and quantified for each sample.[9] The percentage scores were obtained by normalizing to the means of control condition that were defined as being 100%.[11,14]

Statistical Analysis

Data are expressed as mean ± SEM. Mann-Whitney *U* test was used to evaluate the statistical significance of the difference between the groups. Corneal graft survival was assessed by Kaplan-Meier survival curves. The association analysis was performed by the linear mixed model built with the R Studio platform (R Studio Inc., Boston, MA, USA) using the nlme R package. All other statistical analysis was performed with Prism software (GraphPad, La Jolla, CA, USA); *P* < 0.05 was considered significant.

Results

Effect of Itga-9 Blockade on Lymphatic Valvulogenesis After Corneal Transplantation

We first studied the effect of Itga-9 blockade on corneal LG and VG induced by transplantation. Either Itga-9 neutralizing body or isotype control was injected subconjunctivally twice a week starting from the surgery date. As demonstrated in Figure 1A, following the treatment with the Itga-9 blocking antibody, corneal lymphatic vessels contained significantly fewer valves compared with the control condition. Summarized data from repetitive experiments are presented in Figure 1B (left; *P* < 0.05). However, this treatment had no effect on LG, as shown in Figure 1B (right). Our further analysis on the ratio of valve quantity to lymphatic invasion area revealed a significant reduction in this parameter in the treated rather than the control condition (Figure 1C; *P* < 0.05).

Effect of Itga-9 Blockade on Nasal Dominant Distribution of Lymphatic Vessels

Previously, we reported that corneal lymphatic vessels observe a unique nasal dominant distribution pattern in inflammatory
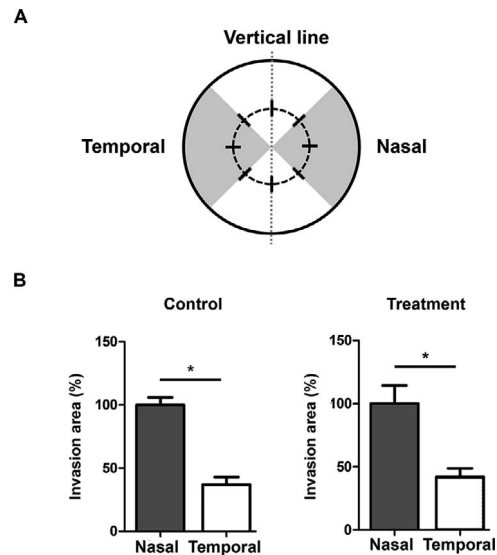

FIGURE 2. Integrin alpha 9 blockade had no effect on polarized distribution of lymphatic vessels after corneal transplantation. (**A**) Schematic illustration of nasal and temporal quadrant areas used for quantification. Eight short lines around the clock indicate sutures placed along the graft border. *Outer solid circle*: limbus. *Inner dotted circle*: graft border. *Vertical line*: separation between the nasal and temporal sides. *Gray-shaded regions*: nasal and temporal areas evaluated. (**B**) Comparative quantification showing significantly greater lymphatic invasion area in the nasal rather than the temporal side in both control and treatment conditions. The experiment was repeated twice with seven mice in control and eight mice in treatment group. **P* < 0.05.

LG.[9,15] To investigate whether this phenomenon also manifests in transplantation-associated LG and whether it is affected by the Itga-9 treatment, we next investigated the effect of Itga-9 blockade on the polarity of LG by comparing the nasal and temporal quadrants, as illustrated in Figure 2A. Our results showed that in both treatment and control groups, lymphatic vessels were more distributed at the nasal side, and Itga-9 blockade had no effect on this polarity of corneal LG (Fig. 2B and Supplementary Figure S1). Our further association analysis using the linear mixed model also confirmed that the polarized distribution of LG was associated only with corneal regions but not with the anti–Itga-9 blockade.

Effect of Itga-9 Blockade on Corneal Graft Survival

To further evaluate the effect of Itga-9 blockade on corneal graft survival, we examined the grafts in both treatment and control groups and evaluated their survival rate twice a week up to 8 weeks after the surgery. As shown in Figure 3, our results showed a significant promotion of graft survival by this treatment. Although graft rejection in both the control and treatment groups started approximately 2.5 weeks after transplantation, a significantly higher percentage of the grafts survived in the treatment group by the end of the 8-week study, as analyzed by the Kaplan-Meier survival curves (*P* < 0.05).

Discussion

In this study, we demonstrated for the first time that Itga-9 is critically involved in corneal transplantation–induced VG, and its molecular blockade can effectively suppress this process. We have also shown that this treatment strategy does not affect
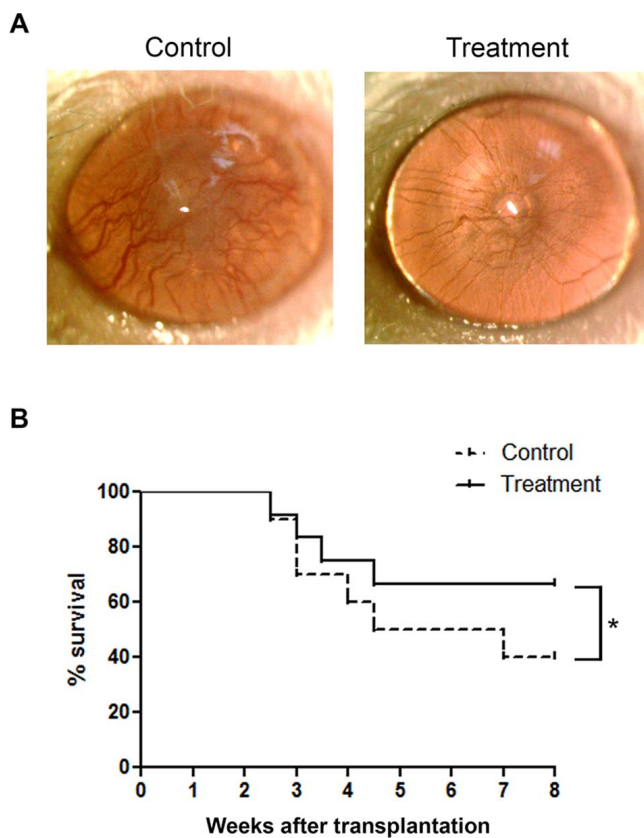
A

B

FIGURE 3. Integrin alpha 9 blockade promoted corneal graft survival. (**A**) Representative images from slit-lamp examination of rejected and survived grafts in control and treatment conditions, respectively. (**B**) Kaplan-Meier survival curves showing significantly higher survival rate in the treatment group. *$P < 0.05$.

corneal LG or its polarity of nasal distribution. More importantly, we have offered the first evidence showing that by reducing the lymphatic valves but not the vessels themselves, we were able to achieve a higher rate of graft survival.

Our finding that Itga-9 blockade suppressed lymphatic valve formation without disturbing the lymphatic vessels in transplantation is consistent with our previous report on a suture-induced inflammation model.[11] It seems that lymphatic valves are more responsive to Itga-9 intervention than lymphatic vessels. This may be explained by the fact that Itga-9 is more highly expressed on the valves than the vessel walls, as shown in Supplementary Figure 2 with a transplanted cornea. The disparity between lymphatic valves and vessels was also observed during development, in which a reduced number of lymphatic valves, but not vessels, were detected in Itga-9 knockout mice.[16] With the treatment regimen used in this study, we did not observe any obvious side effects. For future development of clinical application, it may be possible to achieve the therapeutic effects by using various formats of the antagonists against the Itga-9 pathway, such as neutralizing antibodies or small molecules, which merits further investigation and is beyond the scope of this report.

It is remarkable that prevention of lymphatic valve formation can significantly increase graft survival. This finding indicates a compromise of the immune reflex arc in which the lymphatic pathway serves as the afferent arm.[2,17] It also aligns well with a previous developmental report that Itga-9 knockout mice died shortly after birth from bilateral chylothorax, in which lymphatic vessels were present but displayed compromised integrity.[18] Moreover, we have reported that lymphatic vessels are equipped with valves as they become mature and functional.[8,9] Therefore, by targeting on lymphatic valves, we may have interfered with the maturation process of the lymphatic vessels, rendering them dysfunctional. It would be interesting to check if this strategy also affects other indices of the immune responses involved in transplant rejection, such as delayed-type hypersensitivity, which warrants further investigation.

In summary, this study reveals an important role of lymphatic VG in mediating transplant rejection. It also provides a novel therapeutic strategy to effectively interfere with this pathological process and to improve graft survival. As one of the favorite tools for lymphatic study in general, our results from the cornea may shed some light on the development of new Itga-9–based therapies to treat broader lymphatic and immune diseases in the body.

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