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Posttransplant VEGFR1R2 Trap Eye Drops Inhibit Corneal (Lymph)angiogenesis and Improve Corneal Allograft Survival in Eyes at High Risk of Rejection

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Purpose: To assess whether topical application of VEGFR1R2 Trap after corneal transplantation can impair corneal (lymph)angiogenesis and promote murine corneal allograft survival in eyes at high risk of rejection.

Methods: We used the murine model of suture-induced neovascularization and subsequent keratoplasty in eyes at high risk of rejection, which is an established model for local drug application. After transplantation, the mice were treated with either VEGFR1R2 Trap (aflibercept) or human IgG Fc as eye drops for 2 weeks (three times/d). Deposition of VEGFR1R2 Trap in corneal tissue was detected by immunohistochemistry. Two and 8 weeks after transplantation, corneal (lymph)angiogenesis was assessed morphometrically. Dendritic cells (DCs) and regulatory T cells (Tregs) in the draining lymph nodes (dLNs) were examined by flow cytometry. Allograft survival was determined by corneal graft opacity scores.

Results: Topically applied VEGFR1R2 Trap penetrated into corneal host and graft stroma after keratoplasty in eyes at high risk of rejection. Additional postsurgical corneal hemangiogenesis ($P < 0.0001$) and lymphangiogenesis ($P < 0.01$) as well as infiltrating $CD45^+$ leukocytes ($P < 0.001$) and macrophages ($P < 0.01$) were significantly reduced in the VEGFR1R2 Trap group compared to controls. VEGFR1R2 Trap eye drops significantly decreased the frequency of total $CD11c^+$ DCs ($P < 0.01$), as well as activated $CD11c^+$MHC II^+ DCs ($P < 0.01$) and $CD11c^+CD40^+$ DCs ($P < 0.05$). In contrast, the frequency of $CD200R^+$ regulatory DCs ($P < 0.05$) and Tregs in dLNs ($P < 0.01$) was enhanced. Moreover, long-term allograft survival was also improved ($P < 0.05$).

Conclusions: Temporary, topical application of VEGFR1R2 Trap after corneal transplantation can achieve sufficient anti-VEGF activity, inhibit additional (lymph)angiogenesis, and significantly improve corneal allograft survival in eyes at high risk of rejection.

Translational Relevance: VEGFR1R2 Trap eye drops after transplantation present a new therapeutic option for patients undergoing corneal transplantation and are at high risk of graft rejection.

Introduction

With modern corneal transplantation techniques such as anterior and posterior lamellar keratoplasty, corneal graft survival in patients has improved significantly in recent years, especially in avascular recipient beds at low risk of graft rejection.[1,2] However, not all transplantations can be performed as lamellar keratoplasty, and numerous corneal diseases induce corneal vascularization, resulting in high risk of graft rejection. In fact, in lower- and middle-income countries, more than 70% of transplants are performed in a high-risk setting.[3] Here, immune graft rejection is the main complication, particularly in pathologically vascularized eyes where the 5-year graft survival rates are less than 50%, even with immunosuppressive therapy.[4–6]

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In fact, vascularization of the recipient cornea is one of the most important risk factors for immunologic rejection after keratoplasty in high-risk patients.[7,8] Moreover, surgical trauma by keratoplasty itself can cause additional hemangiogenesis and lymphangiogenesis after transplantation.[9–11] Vascular endothelial growth factor (VEGF) is a key cytokine regulating corneal neovascularization.[3,12–14] This group and others demonstrated that anti-VEGF therapeutic strategies can effectively reduce corneal blood vessel (BV) and lymphatic vessel (LV) growth in both preclinical and clinical trials.[12,14–19] Furthermore, after keratoplasty in eyes at high risk of rejection, modulating corneal hemangiogenesis and lymphangiogenesis by neutralizing VEGF-A applied by intraperitoneal or subconjunctival injections can significantly promote allograft survival.[11,20,21]

The accessibility of the eye makes topical drug application in the form of eye drops feasible. Since this is the least invasive route, eye drops may be a safer option compared to subconjunctival injections and do not result in side effects often linked to systemic application routes.[22–24] Furthermore, eye drops are more likely tolerated by patients. However, due to the cornea's biophysiologic properties and structure, especially the epithelium functioning as a barrier, compounds cannot freely penetrate the cornea.[25] We and others have confirmed that eye drops of VEGFR1R2 Trap (aflibercept) can inhibit inflammatory corneal neovascularization caused by intrastromal sutures or acute chemical burns in preclinical experiments.[15,26–28] The molecular trap designed to bind VEGF-A, VEGF-B, and placental growth factor (PlGF) consists of the ligand-binding regions of the human VEGF receptor 1 (VEGFR1) and VEGF receptor 2 (VEGFR2) fused to the Fc fragment of a human IgG.[29] Its half-life is 4.5 days after intravitreal injection in rabbits.[30] It has been confirmed that VEGFR1R2 Trap has the highest VEGF binding affinity compared to other VEGF inhibitors such as bevacizumab and ranibizumab in both mice and humans.[29] This compound is the most potent antiangiogenic agent currently on the market.[29] We recently showed that preoperative application of VEGFR1R2 Trap can improve corneal graft survival.[26] However, to date, it is unknown whether VEGFR1R2 Trap as eye drops can penetrate the corneal epithelium after keratoplasty in sufficient levels to affect graft survival. Therefore, we analyzed whether topical VEGFR1R2 Trap eye drops can penetrate into the stroma after corneal transplantation, whether the transient topical administration can provide sufficient anti-VEGF activity to inhibit (additional) iatrogenic intrastromal corneal neovascularization, and whether this therapeutic modality affects long-term graft survival in a murine model of keratoplasty in eyes at high risk of rejection.

Materials and Methods

Anesthesia and Animals

Six- to 8-week-old female BALB/c mice and C57BL/6N mice were purchased from Charles River Laboratories (Sulzfeld, Germany). An intraperitoneal injection of ketamine (8 mg/kg; Godecke, Berlin, Germany) and xylazine (0.1 mL/kg; Bayer, Leverkusen, Germany) was administrated to each animal for deep anesthesia before surgery. All animal protocols are in accordance with the Association for Research in Vision and Ophthalmology's Statement on the Use of Animals in Ophthalmic and Vision Research and were authorized by the local animal care and use committee.

Murine Models of Suture-Induced Corneal Neovascularization and Allogeneic Corneal Transplantation in Eyes at High Risk of Rejection

Six- to 8-week-old female BALB/c mice were used in an established model of suture-induced, inflammatory corneal neovascularization.[14] Three interrupted figure-8 sutures (11-0 nylon; Serag-Wiessner, Naila, Germany) were placed in the stroma of the cornea and remained for 14 days to induce neovascularization (Figs. 1A, 1B).

Allogeneic transplantations were performed in the mouse model of corneal transplantation in eyes at high risk of rejection. The procedure has been previously described.[10,31,32] Age-matched C57BL/6 N mice were used as donors for transplantation (Fig. 1C). Subsequently, VEGFR1R2 Trap or human IgG Fc was applied as eye drops (3 µL) at a concentration of 10 mg/mL three times per day for another 2 weeks after keratoplasty. VEGFR1R2 Trap eye drops were prepared by diluting the commercial 40-mg/mL aflibercept formulation (Eylea; Bayer) in sterile Dulbecco's phosphate-buffered saline to 10 mg/mL. Seven days posttransplantation, corneal sutures were removed, and graft opacity was scored by slit-lamp examination once a week until the endpoint of each experiment according to a standardized opacity grading system (0 to +5 levels) as previously described[10] (Fig. 1D). In terms of graft survival, grafts with an opacity score above 2 for at least 2 consecutive weeks were defined as rejected. Mice were euthanized with CO_2 two and eight weeks posttransplantation or other time points as indicated.

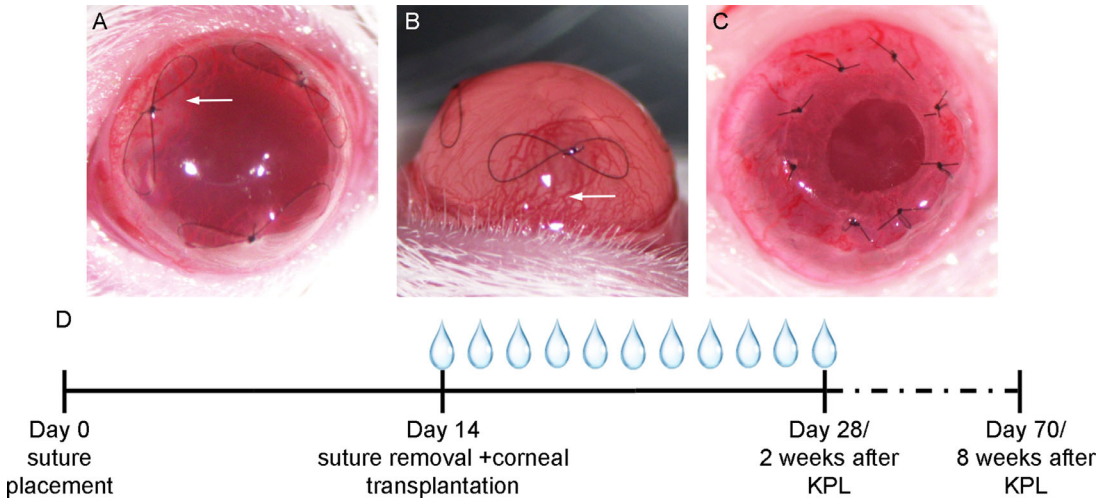

Figure 1. Experimental setup. (A, B) Representative images of corneal recipients in eyes at high risk of rejection induced by suture placement on day 14. (A) Frontal view. (B) Lateral view; *white arrow*: corneal neovascularization. (C) Representative image of keratoplasty on day 14. (D) Schematic overview of the treatment procedure: day 0: placement of intrastromal sutures inducing corneal neovascularization. Day 14: Removal of sutures and corneal transplantation; application of VEGFR1R2 Trap eye drops from day 14 until day 28 posttransplantation three times per day; evaluation of graft survival until day 70 (8 weeks post-KPL). KPL, keratoplasty.

Table. List of Antibodies, Fluorochromes, Clones, and Manufacturers

Antibody	Fluorochrome	Clone	Manufacturer
Rat anti-mouse CD31	FITC	390	BD Bioscience
Rabbit anti-mouse LYVE-1	Unconjugated	Polyclonal	AngioBio
Rat anti-mouse CD45	Alexa Fluor 488	RA3-6B2	BD Bioscience
Hamster anti-mouse CD11c	PE	N418	BioLegend
Rat anti-mouse MHC II	Pacific Blue	M5/114.15.2	BioLegend
Hamster anti-mouse CD40	Alexa Fluor 488	HM40-3	BioLegend
Hamster anti-mouse CD80	APC/Fire	16-10A1	BioLegend
Rat anti-mouse CD86	Alexa Fluor 647	GL-1	BioLegend
Rat anti-mouse CD200R	PE/Cy7	Ba13	BioLegend
Rat anti-mouse CD3	PE/Cy7	17A2	BioLegend
Rat anti-mouse CD8	APC/Cy7	YTS156.7.7	BioLegend
Rat anti-mouse CD4	APC	RM4-5	eBioscience
Rat anti-mouse Foxp3	FITC	FJK-16s	eBioscience
Goat anti-human IgG, Fc_γ	Alexa Fluor 488	Polyclonal	Jackson ImmunoResearch
Goat anti-rabbit IgG (H+L)	Cy3	Polyclonal	Jackson ImmunoResearch

APC, allophycocyanin; CD, cluster of differentiation; Cy, cyanine; FITC, fluorescein isothiocyanate; LYVE-1, lymphatic vessel endothelial hyaluronan receptor 1; PE, phycoerythrin; MHC, major histocompatibility complex.

Immunohistochemistry and Morphologic Assessment of Corneal Wholemounts

Two and 8 weeks after keratoplasty, corneal wholemounts were excised to quantify BVs, LVs, macrophages, and $CD45^+$ cells. Wholemounts were stained with CD31 for BVs and LYVE-1 for LVs (Table). For detection of $CD45^+$ leukocytes, corneal epithelium was removed by incubating the corneas with 20 mM EDTA and then corneas were stained with CD45 (Table). Finally, the percentages of BVs, LVs, $LYVE^+$ macrophages, and $CD45^+$ cells in wholemounts were calculated by Cell^F (Olympus, Münster, Germany) as previously described.[33] Briefly, after

grayscale images of the corneal wholemounts were modified by individual filters, the innermost vessel of the limbal arcade served as the border of the whole cornea, and the graft–host interface defined the corneal graft as well as the recipient bed. The area covered by BVs, LVs, $LYVE^+$ macrophages, and $CD45^+$ cells was determined using threshold analysis. The percentage of vessels or cells was calculated in relation to the area of the whole cornea, either on the graft only or on the host only.

Flow Cytometry Analyses

The ipsilateral lymph nodes corresponding to the corneal wholemounts were excised for cell suspensions 2 weeks and 8 weeks posttransplantation. Afterward, the single-cell suspensions were stained with CD11c-PE, MHC II–pacific blue, CD40-APC, CD80-APC-Cy7, CD86-APC, CD200R-PE-Cy7, CD3-PE-Cy7, CD4-APC, and CD8-APC-Cy7 (Table). Some samples were then fixed and permeabilized using the Fix/Perm kit and stained for Foxp3 (Table). Fluorescence minus 1 was used as the control for the gating setup. Finally, the cells were measured by a Canto II flow cytometer (BD, Franklin Lakes, NJ, USA), and the obtained data were then analyzed using the FlowJo program (version 10.7.1; FlowJo LLC, Ashland, OR, USA).

Detection of VEGFR1R2 Trap in the Naive Cornea and the Cornea After Keratoplasty

After keratoplasty or in naive mice as control, 10 mg/mL VEGFR1R2 Trap was applied as eye drops three times/d (3 μL/time point) for 1 day, 3 days, 1 week, and 2 weeks, respectively ($n = 3$ mice on each time point). Eyeballs were harvested at the indicated time points, embedded in Tissue-Tek O.C.T. Compound (Sakura Finetec, Torrance, CA, USA), and stored at –20°C for further processing.

Cyrosections (6 µm) were blocked with CD16/CD32 mouse Fc Block (BD). Afterward, sections were incubated with goat anti-human IgG Fc (Table) for VEGFR1R2 Trap. Digital images were taken on a fluorescent microscope (BX53; Olympus).

Evaluation of Corneal Epithelial Defects

After transplantation, the size of corneal epithelial defects was determined via 0.1% fluorescein staining. Epithelial defect area was measured by using Cellˆ F software and then calculated in relation to the whole cornea.

Statistical Analyses

Data are presented as mean ± standard deviation (SD) and analyzed by Student's *t*-tests or Kaplan–Meier log-rank test for graft survival using the GraphPad Prism program (version 8; GraphPad Software, La Jolla, CA, USA). Values with $P < 0.05$ were considered statistically significant.

Results

Penetration of Topical VEGFR1R2 Trap Into the Naive Cornea and the Cornea After Transplantation

Corneas were harvested at various time points and tissue sections were stained by fluorescent immunohistochemistry to check whether topically applied VEGFR1R2 Trap can penetrate through the corneal epithelium into the corneal stroma. In naive corneas, VEGFR1R2 Trap displayed no notable penetration into the stroma at 1 day, 3 days, 1 week, and 2 weeks postapplication (Figs. 2A–D). In contrast, VEGFR1R2 Trap applied posttransplantation revealed detectable levels in the stroma of the hosts as well as grafts as early as 24 hours after topical administration and was still detectable 14 days after transplantation (Figs. 2E–L).

Effect of Topical VEGFR1R2 Trap on (Additional) Corneal Hemangiogenesis and Lymphangiogenesis After Transplantation

After demonstrating that VEGFR1R2 eye drops can penetrate the corneal epithelium after transplantation, we investigated whether the absorbed VEGFR1R2 Trap can inhibit corneal hemangiogenesis and lymphangiogenesis induced by transplantation. Therefore, we analyzed the area covered by BVs or LVs 2 and 8 weeks after transplantation. We found that 2 weeks after transplantation, the percentage of corneal BVs and LVs in the whole cornea was significantly reduced in the VEGFR1R2 Trap group (BVs: 26.57% ± 3.44% and LVs: 9.46% ± 1.82%; $n = 10$) in comparison to the IgG Fc control group (BVs: 36.48% ± 3.98%, $P < 0.0001$ and LVs: 12.01% ± 2.90%, $P = 0.0330$; $n = 9$; Figs. 3A–D, 3I). In the area of the graft itself (graft only), hemangiogenesis and lymphangiogenesis were also significantly reduced in the VEGFR1R2 Trap group (BVs: 4.40% ± 2.31% and LVs: 1.71% ± 1.00%; $n = 10$) compared to the IgG Fc control (BVs: 25.06% ± 7.84%, $P < 0.0001$ and LVs: 3.40% ± 1.44%, $P = 0.0078$; $n = 9$; Fig. 3J). In the area of the host

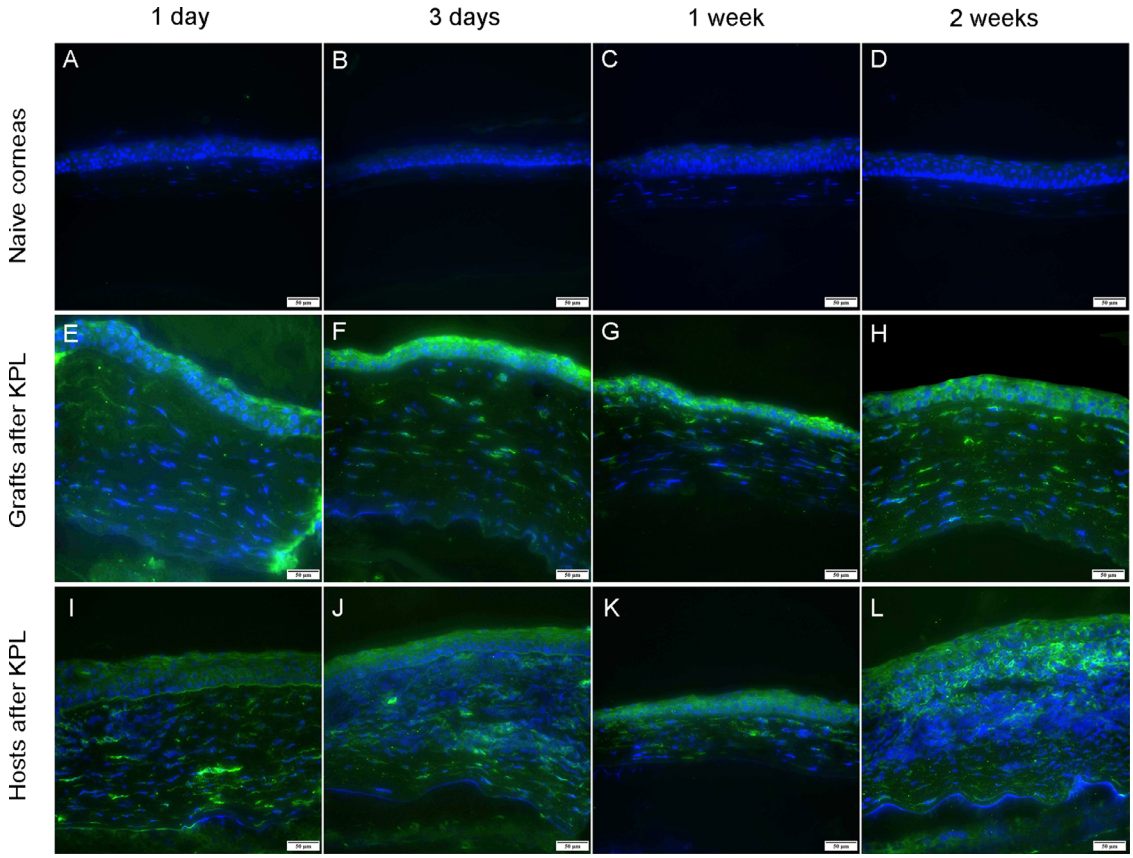

Figure 2. The distribution of topically applied VEGFR1R2 Trap in naive and postsurgical corneas. (A–D) VEGFR1R2 Trap (*green*) was not detectable in the naive corneas at 1 day, 3 days, 1 week, and 2 weeks after application of VEGFR1R2 Trap eye drops (magnification: ×400; *scale bar*: 50 μm). (E–L) VEGFR1R2 Trap (*green*) is present in corneal hosts and in corneal grafts at all time points after keratoplasty (magnification: ×400; *scale bar*: 50 µm) (*blue*: DAPI).

only, significantly lower coverage of BVs and LVs was detected in the VEGFR1R2 Trap group (BVs: 27.39% ± 3.72% and LVs: 9.50% ± 1.83%; $n = 10$) in comparison with the IgG Fc control (BVs: 38.97% ± 4.21%, $P < 0.0001$ and LVs: 12.09% ± 2.93%, $P = 0.0313$; $n = 9$; Fig. 3K).

Eight weeks after transplantation, the inhibitory effect of VEGFR1R2 Trap on hemangiogenesis remained in the VEGFR1R2 Trap group but in the area of the graft only (VEGFR1R2 Trap: 6.16% ± 3.79%, IgG Fc: 12.38% ± 4.52%, $P = 0.0037$; $n = 10$; Fig. 3J). The area covered by BVs in the whole cornea (VEGFR1R2 Trap: 24.28% ± 5.28%, IgG Fc: 25.82% ± 3.44%, $P = 0.4492$; $n = 10$; Fig. 3I), as well as in the host only (VEGFR1R2 Trap: 25.51% ± 5.55%, IgG Fc: 27.58% ± 3.42%, $P = 0.3270$; $n = 10$; Fig. 3K), was similar between both groups at 8 weeks. A reduction of lymphangiogenesis was not detectable 8 weeks postkeratoplasty in any part of the cornea in the VEGFR1R2 Trap-treated group ($P > 0.05$; Figs. 3I, 3J, 3K).

Decrease in Macrophages and CD45$^+$ Cells in Postoperative Corneas

Macrophages play a pivotal role in the recruitment of pathologic corneal lymphatic vessels.[14] Therefore, we analyzed whether the penetration of VEGFR1R2 Trap into the cornea also had an effect on the macrophage infiltration. Two weeks following transplantation, LYVE$^+$ macrophages were significantly decreased in the VEGFR1R2 Trap-treated corneas compared to the IgG Fc control (the whole cornea: 4.73% ± 0.84% [Trap] vs. 6.49% ± 1.60% [control], $P = 0.0071$; the graft only: 4.02% ± 1.32% [Trap] vs. 7.18% ± 2.36% [control], $P = 0.002$; the host only: 4.74% ± 0.84% [Trap] vs. 6.46% ± 1.64% [control], $P = 0.0094$;

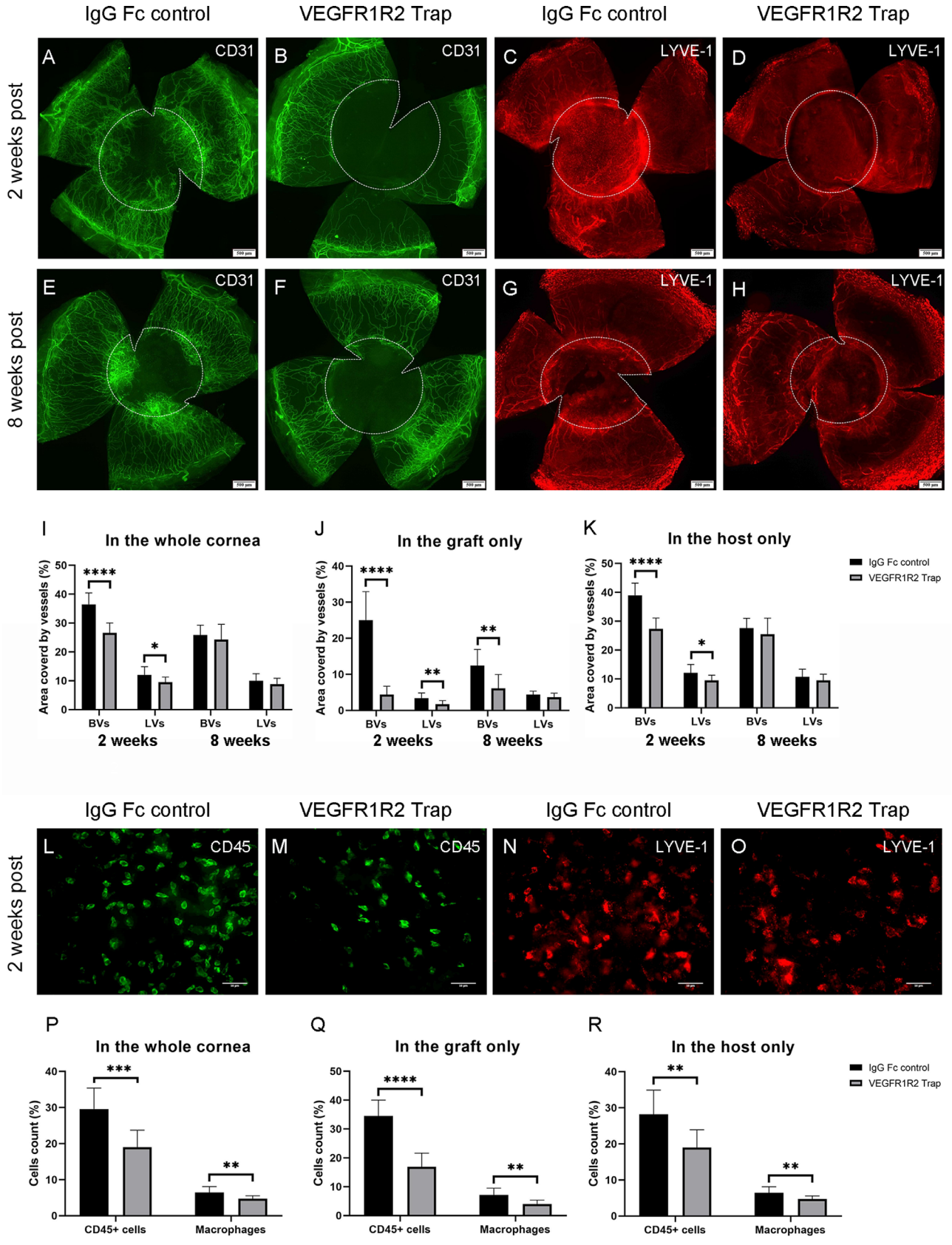

Figure 3. VEGFR1R2 Trap eye drops after keratoplasty inhibit corneal hemangiogenesis, lymphangiogenesis, and immune cell recruitment. (A, B, E, F) Representative corneal wholemounts of control (A, E) and VEGFR1R2 Trap (B, F) treated corneas stained with CD31 for BVs. (C, D, G, H) Representative corneal wholemounts of control (C, G) and VEGFR1R2 Trap (D, H) treated corneas stained with LYVE-1 for LVs 2 and 8 →

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weeks after transplantation (magnification: ×100; *scale bar*: 500 μm; *dotted lines*: graft–host interface). (I) Percentage of BVs and LVs in the whole cornea (the host and graft) 2 and 8 weeks after transplantation in the VEGFR1R2 Trap group compared to IgG Fc control group. (J, K) Percentage of BVs and LVs in the graft only (J) as well as in the host only (K) 2 and 8 weeks after transplantation in the VEGFR1R2 Trap group compared to the IgG Fc control group. (L, M) Representative images of corneal $CD45^+$ cells 2 weeks after KPL (magnification: ×600; *scale bar*: 50 μm). (N, O) Representative images of corneal $LYVE\text{-}1^+$ corneal macrophages 2 weeks after KPL (magnification: ×600; *scale bar*: 50 μm). (P, Q, R) Percentage of $CD45^+$ cells and $LYVE\text{-}1^+$ macrophages in the whole cornea (P), the graft only (Q), and the host only (R) 2 weeks after transplantation in the VEGFR1R2 Trap group compared to the IgG Fc control group (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$).

$n = 9$; Figs. 3N–R). Corneal graft rejection occurs not only due to the presence of blood and lymphatic vessels but also due to the infiltration of immune cells. By using CD45 as a pan-leukocyte marker, we could show that the penetration of VEGFR1R2 Trap into the graft and host tissue after corneal transplantation significantly reduced the percentage of $CD45^+$ cells into the cornea 2 weeks after keratoplasty compared to the IgG Fc group (the whole cornea: 18.99% ± 4.74% [Trap] vs. 29.59% ± 5.82% [control], $P = 0.0004$; the graft only: 16.93% ± 4.72% [Trap] vs. 34.54% ± 5.48% [control], $P = 0.0001$; the host only: 19.02% ± 4.89% [Trap] vs. 28.22% ± 6.69% [control], $P = 0.0031$; $n = 9$; Figs. 3L, 3M, 3P–R).

Effect of VEGFR1R2 Trap on Dendritic Cells and Regulatory T Cells in Draining Lymph Nodes

The penetration of VEGFR1R2 Trap into the graft and host tissue after corneal transplantation also affected the immune cells in the draining lymph nodes. Two weeks after keratoplasty, the frequency of $CD11c^+$ dendritic cells (DCs) in living cells was markedly lower in the VEGFR1R2 Trap group ($CD11c^+$ DCs: 2.09% ± 0.45%; $n = 10$) compared to control ($CD11c^+$ DCs: 3.76% ± 1.73%, $P = 0.0087$; $n = 9$; Figs. 4A, 4B). Moreover, the VEGFR1R2 Trap group featured a significant increase in the frequency of $CD11c^+CD200R^+$ regulatory DCs (18.51% ± 5.58%; $n = 10$) in comparison to the control group (12.49% ± 3.48%, $P = 0.0128$; $n = 9$; Figs. 4C, 4D). No alterations were found in the frequency of $CD11c^+$MHC $II^+/CD40^+/CD80^+/CD86^+$ DCs as well as $CD4^+Foxp3^+$ regulatory T cells (Tregs) between the two groups ($P > 0.05$; Supplementary Fig. 1C).

Eight weeks after surgery, the frequency of $CD11c^+$MHC II^+ DCs (67.89% ± 2.48%; $n = 10$) and $CD11c^+CD40^+$ DCs (49.84% ± 2.73%; $n = 10$) in the draining lymph nodes (dLNs) was significantly reduced in the VEGFR1R2 Trap group compared to control ($CD11c^+$MHC II^+ DCs, 71.39% ± 2.80%, $P = 0.0084$; $CD11c^+CD40^+$ DCs, 54.14% ± 4.08%, $P = 0.0127$; $n = 10$; Figs. 4E–H). However, the frequency of $CD4^+Foxp3^+$ T regulatory cells statistically increased in the VEGFR1R2 Trap group (Tregs, T: 12% ± 0.90%, C: 10.73% ± 1.02%, $P = 0.0082$; $n = 10$; Figs. 4I, 4J). The rate of Tregs in the population of $CD4^+$ T cells was 2983 ± 218 in the VEGFR1R2 Trap group and 2743 ± 312 in the control group. The frequencies of $CD11c^+$ DCs, as well as $CD11c^+CD80^+/CD86^+/CD200R^+$ DCs, were not different between the two groups ($P > 0.05$; Supplementary Fig. 1D).

Effect of Topical VEGFR1R2 Trap on Corneal Epithelial Wound Healing After Keratoplasty in Eyes at High Risk of Rejection

As we observed that VEGFR1R2 trap does not penetrate into naive corneas with an intact epithelium, we evaluated the epithelium defect closure rate after cornel transplantation. We observed that the area of corneal epithelial defects gradually increased from day 1 after keratoplasty and reached a maximum at day 4 posttransplantation in the VEGFR1R2 Trap group and control group. After day 7 (suture removal), the area of corneal epithelial defects dropped down to the level at day 1. This low level was maintained until day 14 posttransplantation in both groups. No statistical differences were noted between the two groups at any time point ($P > 0.05$; Supplementary Fig. 2).

Promotion of Graft Survival in Keratoplasty in Eyes at High Risk of Rejection by Posttransplant VEGF Trap Eye Drops

After demonstrating that VEGFR1R2 eye drops can penetrate the cornea after transplantation and inhibit both corneal hemangiogenesis and lymphangiogenesis and the infiltration of immune cells, we analyzed if these positive effects are also beneficial for graft survival after corneal transplantation in eyes at high risk of rejection. Here, administration of VEGFR1R2 Trap eye drops significantly promoted the graft survival 8 weeks after keratoplasty with a survival rate of 50%, while the survival rate of the control group was 10% ($n = 10$; $P = 0.0138$; Fig. 5; Supplementary Fig. 3).

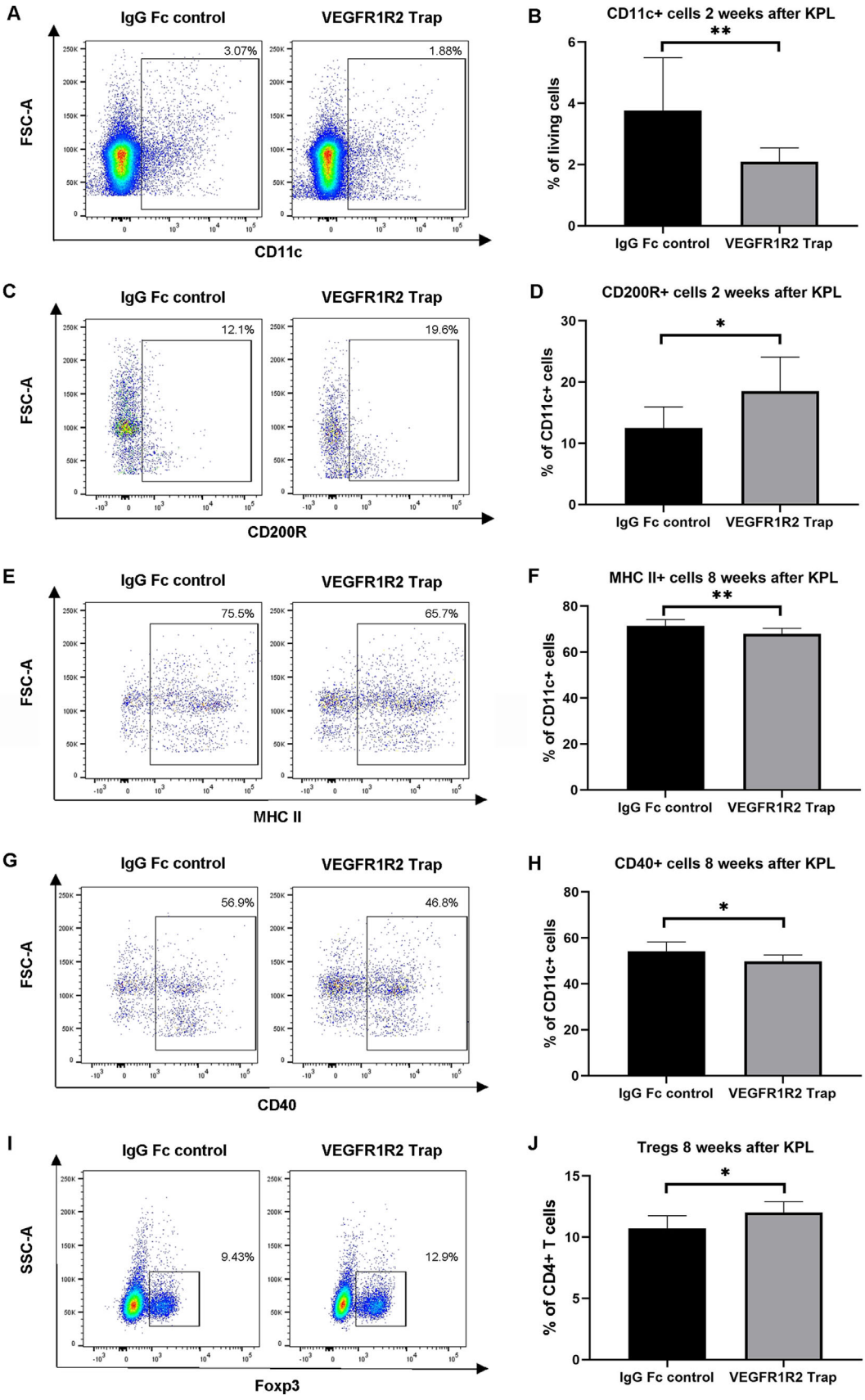

Figure 4. VEGFR1R2 Trap eye drops after keratoplasty led to changes of immune cells in the dLNs. Two weeks after keratoplasty in eyes at high risk of rejection, the frequency of $CD11c^+$ cells was significantly reduced in the VEGFR1R2 Trap group compared to the IgG Fc control group (A, B). Furthermore, the frequency of $CD11c^+CD200R^+$ cells was increased significantly in the VEGFR1R2 Trap group (C, D). Eight weeks →

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posttransplantation, frequencies of CD11c$^+$MHC II$^+$ cells (E, F) and CD11c$^+$CD40$^+$ cells (G, H) in the VEGFR1R2 Trap group were significantly decreased. The frequency of CD4$^+$Foxp3$^+$ Tregs (I, J) was significantly increased in the treatment group 8 weeks after transplantation. *Left panel*: representative dot plots; $n = 10$/group. *$P < 0.05$, **$P < 0.01$. FSC-A, forward scatter area; SSC-A, side scatter area. Gating strategy is shown in Supplementary Figures 1A, 1B.

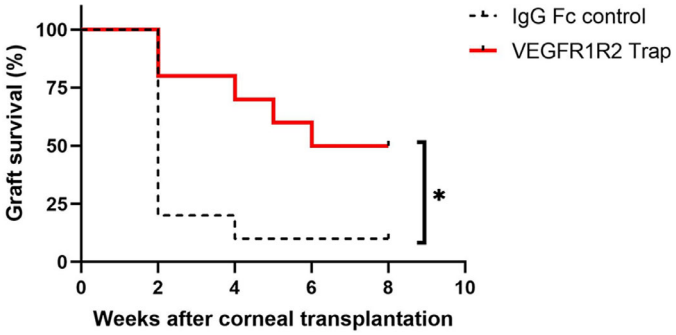

Figure 5. VEGFR1R2 Trap eye drops after corneal transplantation in eyes at high risk of rejection significantly improve corneal graft survival. Corneal grafts from C57BL/6N mice were transplanted into prevascularized BALB/c recipient mice followed by topical application of VEGFR1R2 Trap or human IgG (as control) eye drops for 2 weeks ($n = 10$/group). Weekly grading until 8 weeks after surgery showed an increased graft survival in the VEGFR1R2 Trap group (50%) compared to the control (10%) at 8 weeks after KPL (*$P < 0.05$).

Discussion

This study led to three key observations. First, VEGFR1R2 Trap eye drops applied posttransplantation can effectively penetrate via the corneal epithelium into the stroma. Second, postoperatively topically applied VEGFR1R2 Trap inhibits (additional) corneal hemangiogenesis and lymphangiogenesis and leads to an increase of CD200R$^+$ DCs and Tregs in the dLNs. Third, short-term postoperative application of VEGFR1R2 Trap eye drops improves long-time graft survival in the murine model of keratoplasty in eyes at high risk of rejection.

Eye drops are the least invasive route of ophthalmic drug administration.[34] However, the efficacy of the drug as eye drops may not be sufficient due to the limited bioavailability.[35] It has been demonstrated that the tight junctions of healthy corneal epithelium provide a rate-limiting barrier for the transcorneal transport of compounds, and the barrier can entirely prevent macromolecules from transition ($r > 1$ nm).[25,36] VEGFR1R2 Trap (aflibercept, 115 kDa, $r = 3.70 \pm 0.03$ nm) is a fusion protein consisting of ligand-binding domains of human VEGFR1 and VEGFR2 with the Fc segment of human IgG.[14,37,38] Although topically applied single-chain antibodies or antibody segments of 28 kDa and 67 kDa could penetrate the intact cornea,[39,40] full-sized immunoglobulins are generally considered ineffective due to their large molecule size.[41] In line with these findings, in our study, VEGFR1R2 Trap could not be detected in the normal corneal stroma with intact epithelium even after 14 days of continuous application (Figs. 2A–D). In contrast, after keratoplasty, topically applied VEGFR1R2 Trap could be detected in the host and graft stroma as early as 24 hours after starting the therapy (Figs. 2E–L). It has been shown that the corneal epithelium early after penetrating keratoplasty is abnormal in cell morphology, ultrastructure, and oxygen consumption.[42,43] Also in our study, corneal defects after transplantation could be observed from day 1 postsurgery until day 8 (Supplementary Fig. 2). These factors may contribute to the penetration of VEGFR1R2 Trap into the corneal stroma after keratoplasty.

VEGFR1R2 Trap has been successfully used to inhibit pathologic corneal hemangiogenesis and lymphangiogenesis.[10,15,26] Also, application of VEGFR1R2 Trap eye drops in our study significantly inhibited the ingrowth of new corneal BVs and LVs after transplantation. Notably, at 2 weeks after keratoplasty, which is considered the best time point to assess the immune response to the allograft,[44] CD45$^+$ leukocyte and macrophage frequency was significantly reduced. Since leukocytes participate in protecting the immune system from foreign invaders, the decrease in these cells may facilitate allograft tolerance after transplantation.

Our group has previously reported a reduced frequency of DCs and an increased expression of CD200R in the dLNs at different postoperative periods by preincubating the donor tissue with VEGFR1R2 Trap 1 day before the transplantation.[45] In this study, we show that, in the VEGFR1R2 Trap eye drop–treated group, while CD11c$^+$ DCs reduced and CD200R$^+$ DCs and Tregs increased, the MHC II$^+$ DCs and CD40$^+$ DCs in dLNs were also decreased. In the regional dLNs, the activation of naive T lymphocytes requires their interaction with antigen-presenting cells expressing MHC II$^+$ and costimulatory molecules including CD40, CD80, CD83, and CD86.[4,46] In contrast to enabling T-cell activation, CD200R also has immunoregulatory properties.[47] When injecting CD200R$^+$ DCs into mice, allogeneic tolerance for

skin transplants could be established accompanied by an increased frequency of Tregs.[47] Furthermore, it has been reported that Tregs play a key role in inducing tolerance toward alloantigens.[48] Our group recently showed that corneal grafts preincubated with the immune modulator sCD83[49] as well as with VEGFR1R2 Trap[45] have an increased frequency of $CD200R^+$ DCs together with an increased frequency of Tregs. This indicates an immune modulation of the recipient after transplantation by administration of VEGFR1R2 Trap eye drops and may promote corneal allograft survival.

In this study, the application of VEGFR1R2 Trap eye drops could increase the long-term allograft survival in eyes at high risk of rejection up to 50%. In previous studies using VEGFR1R2 Trap in the model of keratoplasty in eyes at high risk of rejection after transplantation, the survival rate was 23% by intraperitoneal injection.[11] Studies by others have shown that subconjunctival injection of VEGFR1R2 Trap could improve the 8-week allograft survival to 72%.[21] One reason for the described differences could be the variations in the treatment regimens. In our study, VEGFR1R2 Trap was applied transiently posttransplantation for 2 weeks by eye drops, whereas Bachmann et al.[11] performed intraperitoneal injections. In contrast, the subconjunctival injections of VEGFR1R2 Trap by Dohlman et al.[21] were administrated throughout the 8-week follow-up period after transplantation. The continued postoperative treatment with VEGFR1R2 Trap may contribute to the increased allograft survival. Nevertheless, repeated subconjunctival injections increase the risk of trauma and infection.[22–24] Furthermore, another VEGF inhibitor, bevacizumab, was also used to study the effects of topical and subconjunctival application on the graft survival in the murine model of keratoplasty in a high-risk context.[20] The results demonstrated that transient administration of bevacizumab after transplantation by subconjunctival injection could promote allograft survival (33%), whereas eye drops could not (0%).[20] VEGFR1R2 Trap (115 kDa) with a smaller molecule than bevacizumab (149 kDa) could therefore potentially penetrate more efficiently into the neovascularized cornea, when applied as eye drops.[27] Moreover, the affinity of VEGFR1R2 Trap to VEGF-A, which is about 120 times higher than bevacizumab,[29] further contributes to the differences of long-term graft survival between the two treatments. Therefore, further studies should be carried out to assess the optimal dosage and frequency of VEGFR1R2 Trap eye drop administration to achieve the optimum anti-VEGF activity after high-risk keratoplasty and thereby further increase allograft survival.

Moreover, our study confirms that even in recipient beds with established neovascularization and at high risk of rejection, the inhibition of posttransplant additional hemangiogenesis and lymphangiogenesis can significantly promote corneal graft survival.[11] That may allow for transfer of this concept (promotion of graft survival by posttransplantation anti-VEGF therapy) to other sites of transplantation in the body that are physiologically prevascularized.[3]

It has been reported that continuous local anti-VEGF therapies can cause side effects, including delayed corneal epithelial healing and cornea melt.[50] However, in our study, we did not observe a delayed epithelial wound healing after high-risk keratoplasty by VEGFR1R2 Trap eye drops. This is consistent with our previous studies in which VEGFR1R2 Trap ophthalmic solution also did not affect corneal reepithelialization.[15] Nevertheless, careful follow-up studies of any adverse effects of long-term VEGFR1R2 Trap application are necessary.

Taken together, our study demonstrated that temporary topical administration of VEGFR1R2 Trap eye drops after corneal transplantation can achieve sufficient anti-VEGF activity. Thereby, VEGFR1R2 Trap eye drops inhibit hemangiogenesis, lymphangiogenesis, and the recruitment of $CD45^+$ cells in the cornea, reduce DCs in the dLNs and modulate their phenotype, increase Tregs in dLNs, and improve subsequent long-term allograft survival in eyes at high risk of rejection. This approach could be a promising therapeutic strategy to promote graft survival in eyes at high risk of rejection and could also be useful at transplantation of other organs.

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