Article

VEGF Trap$_{R1R2}$ Suspended in the Semifluorinated Alkane F6H8 Inhibits Inflammatory Corneal Hem- and Lymphangiogenesis

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Purpose: Semifluorinated alkanes (SFAs) are used at the ocular surface as lubricants or vehicles for drugs. The purpose of this study was to test the effect of vascular endothelial growth factor (VEGF) Trap$_{R1R2}$ suspended in the SFA perfluorohexyloctane (Trap/F6H8) on corneal neovascularization.

Methods: Suture placement was used to induce inflammatory corneal neovascularization in mice. Treatment groups were: Trap/F6H8, VEGF Trap$_{R1R2}$ as aqueous formulation dissolved in phosphate buffer (Trap), F6H8, and phosphate buffer (controls). Eye drops were applied 3×/daily for 2 weeks. Afterward, corneas were stained with CD31 and LYVE-1 to analyze corneal hem- and lymphangiogenesis. To investigate the effect of on inflammatory cell recruitment, corneal CD45+ cells were quantified. In addition, epithelial wound closure after debridement was assessed by corneal fluorescein staining.

Results: Trap/F6H8 was as effective as Trap in inhibiting corneal hemangiogenesis and lymphangiogenesis after 2 weeks of treatment. After 3 days of treatment, Trap/F6H8 was even more effective than Trap in inhibiting corneal hemangiogenesis. Both treatment groups (Trap/F6H8 and Trap) significantly reduced corneal CD45+ cell recruitment. Epithelial closure after debridement was unaffected by Trap/F6H8 or Trap.

Conclusions: In this study, we demonstrate that F6H8 is a potential carrier for VEGF Trap$_{R1R2}$ to topically treat corneal neovascularization. Our findings might open new treatment avenues for local anti-angiogenic therapy at the cornea, as F6H8 is already approved for the usage at the ocular surface.

Translational Relevance: With this study we show for the first time that SFAs can serve as carriers for anti-angiogenic drugs at the ocular surface.

Introduction

The healthy cornea is avascular and alymphatic. However, corneal neovascularization can occur as a result of severe inflammation, infection, trauma, or degenerative disorders. The presence of corneal neovascularization prior to corneal transplantation (keratoplasty) is one of the most important risk factors for the development of corneal transplant rejection.[1,2] In fact, grafts transplanted into an avascular recipient (so called low-risk keratoplasty) show a 5-year survival rate of 80 to 90%, whereas grafts transplanted into prevascularized recipients (so called high-risk keratoplasty) are rejected in more than 50%, despite immunosuppressive therapy.[3–5] Preclinical evidence suggests

that particularly lymphatic vessels play an important role in the induction of corneal transplant rejection, dry eye disease, and allergic responses at the ocular surface.[6–8] Lymphatic vessels seem to facilitate access of antigen-presenting cells to regional lymph nodes, where accelerated antigen sensitization occurs.[4] Thus, over the past decades, anti-hem- and lymphangiogenic treatment strategies have been developed, and the benefit of this approach has already been shown in the preclinical and clinical setting.[6,8–16]

The members of the vascular endothelial growth factor (VEGF) family are essential growth and survival factors involved in pathological hem- and lymphangiogenesis.[17] In particular VEGF-A is one of the most important growth factors for corneal hem- and lymphangiogenesis. VEGF-A can bind to its receptors VEGFR1 and VEGFR2 on blood and lymphatic endothelial cells, inducing endothelial cell proliferation.[17] In addition, VEGF-A may also mediate chemotactic effects by binding to VEGFR1 expressed on macrophages, thereby perpetuating an inflammatory hem- and lymphangiogenic response in the cornea[18] Thus, VEGF-A has emerged as the prime target for the inhibition of progressive corneal neovascularization.[19–22] VEGF Trap$_{R1R2}$ (Aflibercept), is a 115 kDa fusion protein comprising of the ligand-binding domains of VEGFR1 and VEGFR2 coupled to the Fc portion of IgG1.[23] VEGF Trap$_{R1R2}$ binds VEGF-A, VEGF-B, and PlGF and it has been demonstrated that VEGF Trap$_{R1R2}$ inhibits corneal hem- and lymphangiogenesis in a mouse model of inflammatory corneal neovascularization.[18,24] Systemic application of VEGF Trap$_{R1R2}$ almost completely inhibited corneal hem- and lymphangiogenesis.[18] Topical treatment also showed marked neovascularization reduction, although with reduced efficacy when compared to the systemic application route.[24] Thus, an improvement of local efficacy for the treatment of corneal neovascularization is desirable, as systemic VEGF blockade might lead to potential side effects. However, no direct anti-VEGF therapy for corneal neovascularization is approved yet.

The semifluorinated alkane (SFA) perfluorohexyloctane (F6H8) is a water-free, inert, nontoxic, and amphiphilic liquid with low surface and interface tension and high biocompatibility.[25] Recently, F6H8 has been tested as treatment for dry eye disease (NovaTears).[26] The advantage of F6H8 eye drops is that F6H8 does not require any preservatives or surfactants. F6H8 does not cause any toxic effects at the cornea and has further been shown to significantly improve clinical signs and symptoms of patients with dry eye disease.[26–28] Currently, the properties of SFAs as vehicles for topical drugs are under investigation. The low surface tension leads to increased spreadability across the corneal surface, which together with the longer corneal residence time compared to aqueous eye drops results in higher bioavailability of active pharmaceutical ingredients (API).[29,30] In addition, protein drugs, such as VEGF Trap$_{R1R2}$ often present a formulation challenge due to the chemical and physical instability of these biologics, which tend to agglomerate, aggregate, and/or denaturate during long-term storage. It has been shown that protein API in SFAs are more stable at room and elevated temperatures compared to aqueous formulations.[31,32]

Based on these previous findings, we investigated the inhibitory effect of VEGF Trap$_{R1R2}$ suspended in F6H8 (Trap/F6H8) on inflammatory corneal neovascularization.

Methods

Preparation of VEGF Trap$_{R1R2}$ Compositions

The water-free VEGF Trap$_{R1R2}$ suspension in F6H8 (Novaliq GmbH, Heidelberg, Germany) was prepared from the aqueous commercial 25 mg/mL aflibercept formulation (Zaltrap; Sanofi Genezyme). After dilution with 5 mM sodium phosphate buffer (pH 6.2) to obtain a total solid content of 4 to 5% (w/v), VEGF Trap$_{R1R2}$ protein powder material was prepared by spray-drying utilizing a Büchi B290 Mini Spray Dryer. The water-free VEGF Trap$_{R1R2}$ suspension in F6H8 was then obtained by vortexing of the spray-dried protein powder material in F6H8 and subsequent sonication in an ultrasound bath filled with ice water, to receive 5 mg/mL VEGF Trap$_{R1R2}$ suspended in F6H8 (Trap/F6H8). VEGF Trap$_{R1R2}$ dissolved in phosphate buffer (Trap) was prepared by diluting the commercial 25 mg/mL aflibercept formulation (Zaltrap; Sanofi Genezyme) in sterile 10 mM sodium phosphate buffer (pH 6.2) to the desired final protein concentration of 5 mg/mL.

Mice and Anesthesia

All experimental procedures were approved by the local animal care and use committee and conform to the Association for Research in Vision and Ophthalmology's Statement for the Use of Animals in Ophthalmology and Vision Research. Female BALB/C mice, purchased from Charles River Laboratories, Sulzfeld, Germany (aged 6–8 weeks) were used in all experiments. Prior to surgery, a mixture of ketamine hydrochloride (100 mg/kg) and xylazine (10 mg/kg) was intraperitoneally injected to deeply anesthetize the mice.

Suture-Induced Corneal Neovascularization Assay and Treatment Regimes

The mouse model of suture-induced inflammatory neovascularization was performed as previously described.[33,34] Three 11-0 nylon sutures (Serag Wiessner, Naila, Germany) were inserted intrastromally on the right eye with two stromal incursions extending over 120 degrees of corneal circumference each. The outer point of suture placement was chosen near the limbus and the inner suture point was placed near the center of cornea equidistant from the limbus to obtain standardized angiogenic responses. Mice were divided into four groups ($n = 10$ per group): VEGF Trap$_{R1R2}$ suspended in F6H8 (Trap/F6H8), VEGF Trap$_{R1R2}$ dissolved in phosphate buffer (Trap), F6H8, and phosphate buffer. Right after suturing, drops were applied three times per day. After 2 weeks of topical administration, corneas were harvested and wholemounts were stained for CD31 and LYVE-1 to quantify corneal hem- and lymphangiogenesis (see below). In indicated experiments, eye drops were applied 3 times per day for 3 days. Afterward, corneas were harvested to quantify CD45+ cells and to analyze corneal hem- and lymphangiogenesis.

Immunohistochemistry and Morphological Analysis of Corneal Wholemounts

The blood and lymphatic vessels in corneal wholemounts were double stained as described previously.[33,35] Excised corneas were rinsed in PBS and then fixed in acetone for 20 minutes. After washing in PBS 3 times, and blocking with 2% bovine serum albumin (BSA) in PBS, the corneas were stained overnight (in dark, at 4°C) with rabbit anti-mouse LYVE-1 (1:200; AngioBio, Del Mar, CA, USA) for lymphatic vessels and with an FITC-conjugated rat anti-mouse CD31 antibody (1:100; BD Pharmingen, BD Biosciences, San Jose, CA) for blood vessels. On the next day, a goat-anti-rabbit Cy3-conjugated secondary antibody (1: 100; Dianova) was added to detect LYVE-1. Finally, samples were mounted on slides using fluorescence mounting media (Sigma). In indicated experiments, corneas were stained with Alexa Fluor 488 rat anti-mouse CD45 receptor antibody (BD Pharmingen, Heidelberg, Germany) to quantify CD45+ cells.

Stained wholemount images were assembled automatically from 9 to 12 images taken at ×100 magnification with a fluorescence microscope (BX53; Olympus Optical Co., Hamburg, Germany). Afterward, the areas covered with blood and lymphatic vessels (or CD45+ cells) were detected with an algorithm established in the image analyzing program CellˆF (Olympus Soft Imaging Solutions GmbH, Münster, Germany), as previously described.[33]

Measurement of Epithelial Defect Size

To evaluate the effect of Trap/F6H8 on corneal epithelial wound closure, corneas of five mice per group were de-epithelialized. Application of eye drops (3 times/day) was started on the next day. Mice were examined daily to assess corneal re-epithelialization. Corneal epithelial defect size was determined via 0.1% fluorescein staining. The epithelial defect area was measured by CellˆF software and then calculated in relation to the whole corneal area. Complete re-epithelialization was defined when there was no detectable fluorescein staining in the cornea.

Statistical Analyses

Statistical analyses were performed with Prism 8 version 8.0.2 (GraphPad Software, San Diego, CA). Statistical significance between two groups was determined using Student's *t*-test or ANOVA for multiple groups. Data are reported as mean ± standard deviation (SD). Values with $P < 0.05$ were considered statistically significant. Graphs were drawn using Prism version 8.

Results

Stability of the VEGF Trap$_{R1R2}$ Suspension in F6H8

Samples of the water-free VEGF Trap$_{R1R2}$ suspension in F6H8 were subjected to a stability study for 3 months at 2 to 8°C and 25°C / 60% relative humidity (RH). Testing two replicas at each time point (0, 1, and 3 months), the samples were assayed for concentration, protein aggregation, and fragmentation, as well as protein activity. Analysis by UV-absorption, SEC-MALS (Size Exclusion Chromatography Multi Angle Light Scattering) and ELISA (Aflibercept ELISA, ImmunoGuide) revealed that the VEGF Trap$_{R1R2}$ suspension in F6H8 is exceptionally stable with respect to assay, aggregation, and activity, even when stored at ambient temperature for at least 3 months (Table).

Table. Stability of the VEGF Trap$_{R1R2}$ Suspension in F6H8

Temperature	Test	Method	0 mo	1 mo	3 mo
2–8°C	Assay	UV – Absorption	4.01 mg/mL	4.11 mg/mL	4.28 mg/mL
	Activity	ELISA	103.6%	n. t.	90.8%
	Aggregation	SEC-MALS	<LOD	1.1%	0.25%
25°C / 65% RH	Assay	UV – Absorption	4.01 mg/mL	4.19 mg/mL	4.25 mg/mL
	Activity	ELISA	103.6%	n. t.	96.5%
	Aggregation	SEC-MALS	<LOD	1.8%	0.5%

Samples of the water-free VEGF Trap$_{R1R2}$ suspension in F6H8 were assayed for concentration, protein aggregation and fragmentation, as well as protein activity at 0, 1, and 3 months. The VEGF Trap$_{R1R2}$ suspension in F6H8 was exceptionally stable in respect to assay, aggregation, and activity, even when stored at ambient temperature for at least 3 months (two replicas at each time point).

VEGF Trap$_{R1R2}$ Suspended in F6H8 is as Effective as the Aqueous Formulation of VEGF Trap$_{R1R2}$ in Inhibiting Corneal Hem- and Lymphangiogenesis

To investigate the effect of VEGF Trap$_{R1R2}$ suspended in F6H8 (Trap/F6H8) on inhibition of hem- and lymphangiogenesis, eye drops at a concentration of 5 mg/mL were applied in the suture-induced corneal inflammatory neovascularization model. Quantitative analysis of the vascularized corneal area at day 14 after suturing showed that Trap/F6H8 is as effective as the aqueous commercial formulation of VEGF Trap$_{R1R2}$ (Trap) in inhibiting corneal hemangiogenesis and lymphangiogenesis (Fig. 1). When compared with the control groups F6H8 and phosphate buffer, both treatment groups significantly inhibited blood vessels (mean vascularized area ± SD in Trap/F6H8: 13.55% ± 4.83%; Trap: 13.73% ± 6.65%; F6H8: 19.58% ± 4.64%; phosphate buffer: 19.23% ± 5.50%; $P < 0.05$) and lymphatic vessels (mean vascularized area ± SD in Trap/F6H8: 1.71% ± 0.81%; Trap: 2.03% ± 0.81%; F6H8: 3.19% ± 1.19%; phosphate buffer: 3.47% ± 0.99; $P < 0.01\%$).

VEGF Trap$_{R1R2}$ Suspended in F6H8 is More Effective Than the Aqueous Formulation of VEGF Trap$_{R1R2}$ in Inhibiting Corneal Hemangiogenesis at Early Time Points

To further compare both treatments (Trap/F6H8 and Trap) in inhibiting blood and lymphatic vessels at an earlier time point, corneas were harvested 3 days after suture placement. Hemangiogenesis was significantly inhibited in both treatment groups compared with controls (mean vascularized area ± SD in Trap/F6H8: 2.25% ± 2.17%; Trap: 4.85% ± 1.49%; F6H8: 7.39% ± 1.25%; phosphate buffer: 8.81% ±

1.25%; $P < 0.01$). Of note, Trap/F6H8 showed a significantly better efficacy of hemangiogenesis inhibition at early time points in comparison to Trap ($P < 0.05$; Fig. 2A). Both Trap/F6H8 and Trap did not reach significant inhibition of lymphangiogenesis at this early time point (Fig. 2B). However, analysis of additional vessel morphometric parameters, such as lymphatic vessel sprouts (mean number of sprouts ± SD in Trap/F6H8: 12.00 ± 3.93; Trap: 12.60 ± 2.50; F6H8: 17.75 ± 6.52; phosphate buffer: 23.00 ± 5.16; $P < 0.05$), lymphatic vessel branching (mean number of branches ± SD in Trap/F6H8: 6.56 ± 2.51; Trap: 6.30 ± 2.41; F6H8: 12.00 ± 4.28; phosphate buffer: 14.80 ± 3.94; $P < 0.05$) and lymphatic vessel end points (mean number of end points ± SD in Trap/F6H8: 17.11 ± 3.33; Trap: 18.40 ± 3.24; F6H8: 26.88 ± 8.50; phosphate buffer: 32.10 ± 7.16; $P < 0.05$) revealed significant inhibitory effects when compared to controls, but without a significant difference between the treatment groups.

VEGF Trap$_{R1R2}$ Suspended in F6H8 Reduces CD45$^+$ Inflammatory Cells in Inflamed Corneas

Leukocytes are important mediators of inflammatory corneal hem- and lymphangiogenesis. In addition, it has previously been shown that blockade of VEGF-A leads to an inhibition of corneal hem- and lymphangiogenesis that is at least partially attributable to the reduction of leukocyte recruitment.[18] Therefore, to analyze whether treatment groups show a potentially differential inhibition of leukocyte recruitment, we additionally quantified leukocytes 3 days after treatment with Trap/F6H8, Trap, or controls. Corneas treated with Trap/F6H8 or Trap showed significantly reduced CD45$^+$ cell quantity at this early time point: treatment groups had a significant reduction of
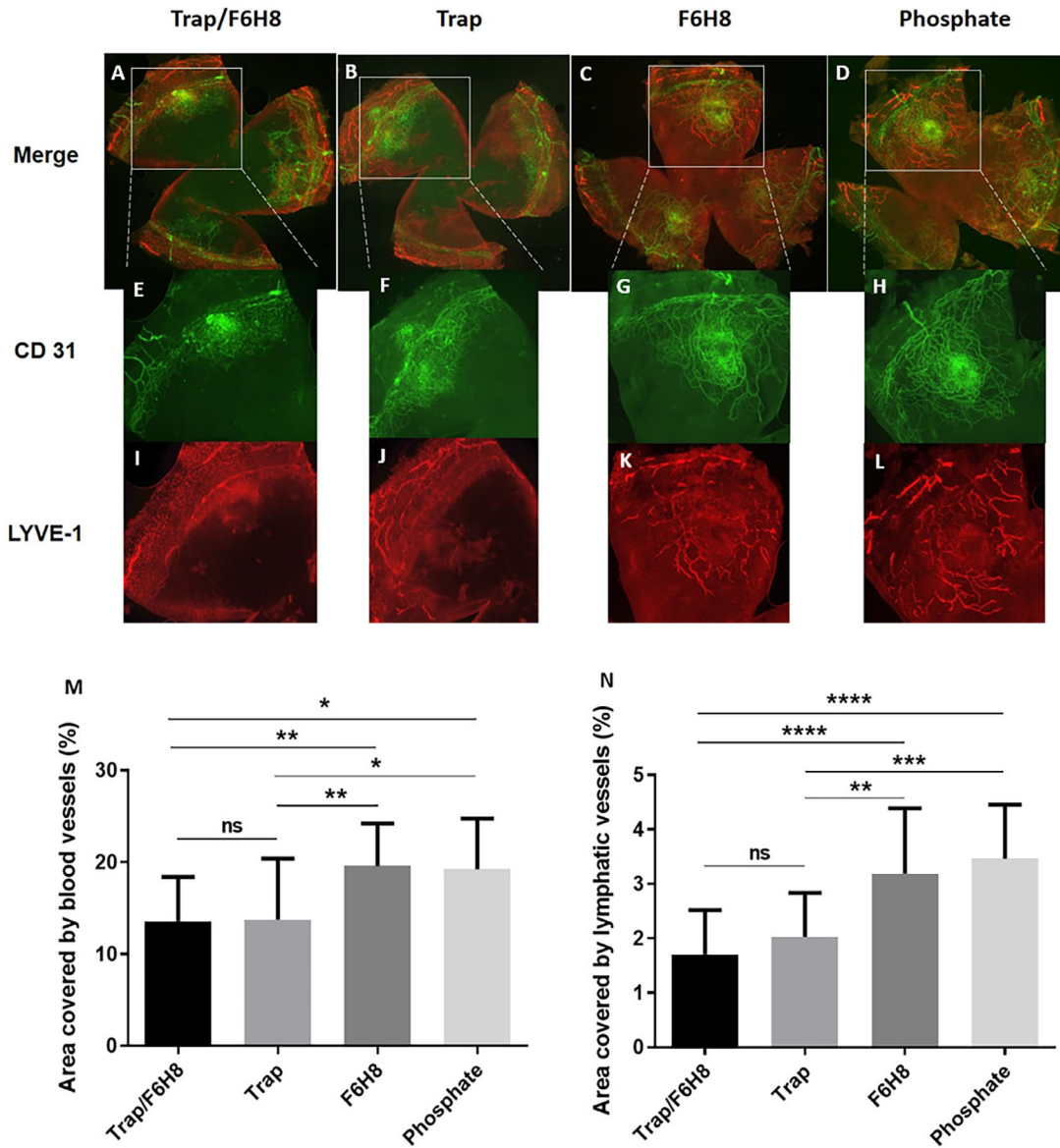
Figure 1. **Inhibition of corneal blood and lymphatic vessels after topical administration of Trap/F6H8 for 14 days**. Wholemount immunohistochemistry staining (**A–D**) was carried out to quantify the corneal area covered by blood and lymphatic vessels. Blood vessels (*green*) were stained with CD31 (**E–H**: blood vessels) and lymphatic vessels (*red*) were stained with LYVE-1 (**I–L**: lymphatic vessels). Blood and lymphatic vessels were significantly inhibited in groups treated with Trap/F6H8 and Trap compared to control groups F6H8 and phosphate buffer (n = 10 each group; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$). Between the two treatment groups, there was no significant difference in the inhibition of hem- or lymphangiogenesis (n = 10 each group, ns = $P > 0.05$).

inflammatory cells in comparison to the phosphate buffer control group (mean area covered by leukocytes $\pm$ SD in Trap/F6H8: 69.93% $\pm$ 18.72%; Trap: 72.81% $\pm$ 15.28%; phosphate buffer: 100% $\pm$ 21.02%, $P < 0.01$ and $P < 0.05$, respectively). The F6H8 group also had more CD45$^+$ cells than the treatment groups (mean area covered by leukocytes $\pm$ SD in F6H8: 85.09% $\pm$ 21.88%), albeit without statistical difference (Fig. 3).

Effect of VEGF Trap$_{R1R2}$ Suspended in F6H8 on Corneal Re-Epithelialization

To evaluate the safety and the effect of Trap/F6H8 on corneal epithelial wound closure, corneal epithelial defects sparing the limbal area were created as described previously.[14] After epithelial debridement, eye drops were applied three times per day and corneal epithelial wound closure was assessed by corneal
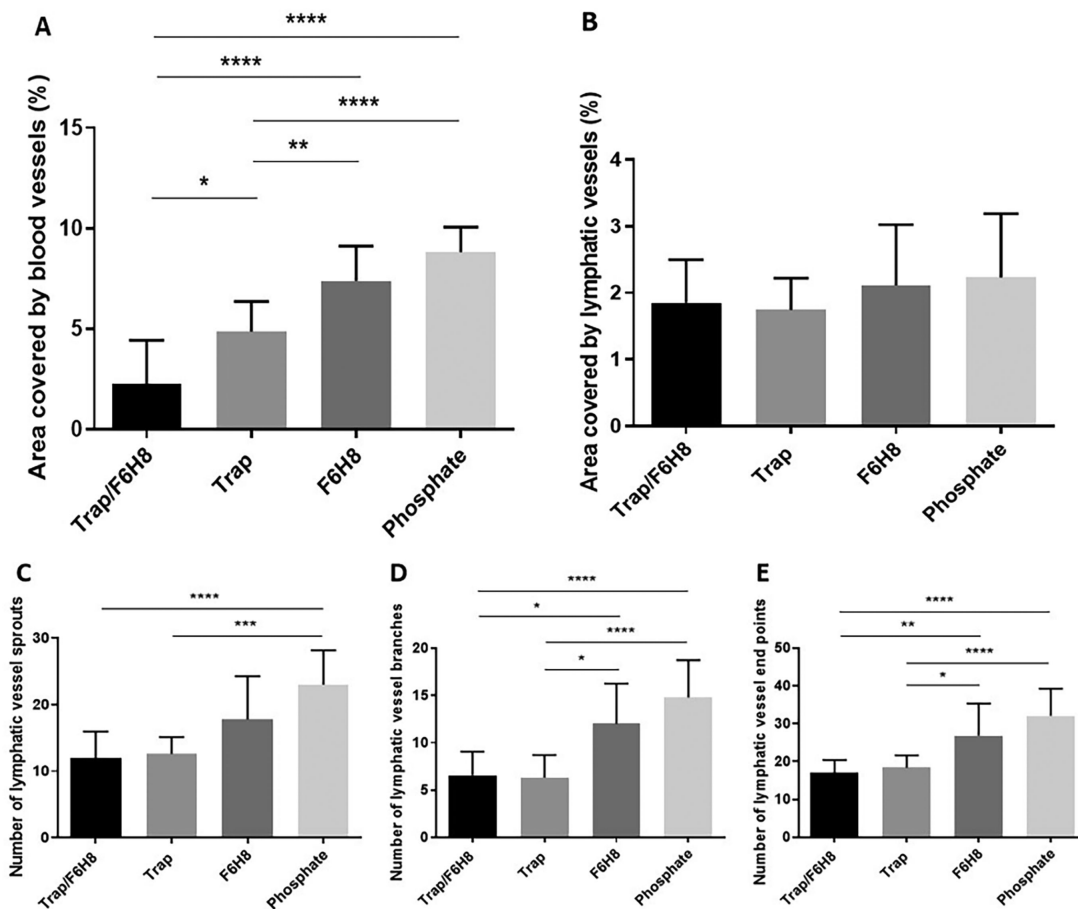
Figure 2. **Effect of Trap/F6H8 in inhibiting corneal hem- and lymphangiogenesis after 3 days.** Quantitative analysis at day 3 revealed a significant inhibition of the ingrowth of blood vessels in both treatment groups (Trap/F6H8 and Trap) compared to control groups (F6H8 and phosphate buffer (**A**). Trap/F6H8 was more effective than Trap at this time point in inhibiting corneal hemangiogenesis ($P < 0.05$). Whereas Trap/F6H8 and Trap had effects on hemangiogenesis, both did not significantly inhibit lymphangiogenesis at this time point (**B**). However, the number of lymphatic vessel sprouts, branches, and end points were significantly reduced in both treatment groups in comparison to control groups (**C–E**) ($n = 10$ each group, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$).

fluorescein staining. There was no significant difference in epithelial wound closure between all groups (Fig. 4). All corneal defects completely healed 8 days after corneal debridement, indicating that Trap/F6H8 does not affect corneal re-epithelialization.

Discussion

The main findings of our work are: (1) when compared to the aqueous commercially available VEGF Trap$_{R1R2}$ (Trap) formulation, VEGF Trap$_{R1R2}$ suspended in F6H8 (Trap/F6H8) has at least a comparable anti-hem- and anti-lymphangiogenic effect in the cornea; (2) treatment with Trap/F6H8 reduces corneal leukocyte infiltration; and (3) Trap/F6H8 does

not affect corneal re-epithelialization, indicating that Trap/F6H8 is nontoxic and safe when applied to the ocular surface.

Trap/F6H8 applied as eye drops strongly inhibited corneal hem- and lymphangiogenesis after 14 days of treatment. The inhibition of hemangiogenesis after 3 days of treatment was even more pronounced in Trap/F6H8-treated eyes when compared to Trap-treated eyes. This finding is in line with previous findings on the use of SFAs as drug carriers at the ocular surface: Gehlsen et al. have shown experimentally that cyclosporin A dissolved in SFA is equally effective when compared to a commercially available cyclosporin A formulation, but with a significantly faster therapeutic response in reducing signs of dry-eye disease.[36] This has been validated in one phase II and one phase IIb/III clinical trial, where the
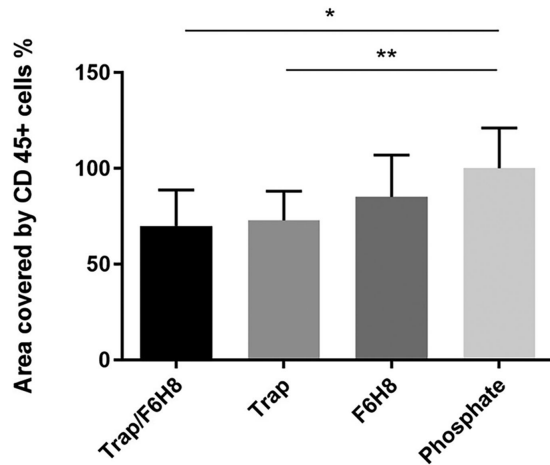
Figure 3. **Reduction of CD45$^+$ inflammatory cells in inflamed corneas after Trap/F6H8 treatment**. Corneal CD45$^+$ cells were significantly lower at day three in the Trap/F6H8 group and the Trap group compared to the control groups. There was no significant difference between the two treatment groups ($n = 5$ each group, $*P < 0.05$, $**P < 0.01$).

Figure 4. **Corneal epithelial wound closure after topical application of Trap/F6H8**. (**A**) Representative fluorescein staining images illustrate epithelial defect healing from day 0 (time of corneal epithelial removal) until day 8 among groups treated with Trap/F6H8, Trap, or control groups (F6H8 or phosphate buffer). (**B**) All corneas achieved complete re-epithelialization 8 days after corneal abrasion with no significant difference between all groups from day 0 to day 8.

SFA-based cyclosporin A formulation showed significant improvements in corneal staining as early as 2 weeks after treatment initiation.[37,38]

In terms of corneal neovascularization, it has previously been shown that corneal penetration of antibodies directed against VEGF is limited when the corneal epithelium is intact and that drug penetration is increased in inflamed and neovascularized corneas.[39] This might indicate that the barrier function of the epithelium is reduced during the inflammatory and neovascular response. However, it is likely that some epithelial barrier function might still be present especially in the very early phase after injury. Our current results indicate that the use of SFAs as vehicle improves early anti-hemangiogenic efficacy of Trap. Thus, SFAs as drug carriers seem to increase bioavailability especially in the early phase after injury, which might be of importance in the treatment of active, progressive corneal neovascularization.

Other benefits of SFA-formulations, especially for proteins and antibodies, include ready-to-use eye drops with improved stability at room temperature and above and enhanced API uptake. The suspended particles deliver high localized API concentrations and increase residence time on the cornea.[29]

As already mentioned, SFAs have recently attracted considerable attention as eye drop therapies for the treatment of dry eye disease. Here, SFAs have been shown to improve tear film break up time, corneal fluorescein staining, and subjective symptoms of affected patients.[26,28,37,38,40] Thus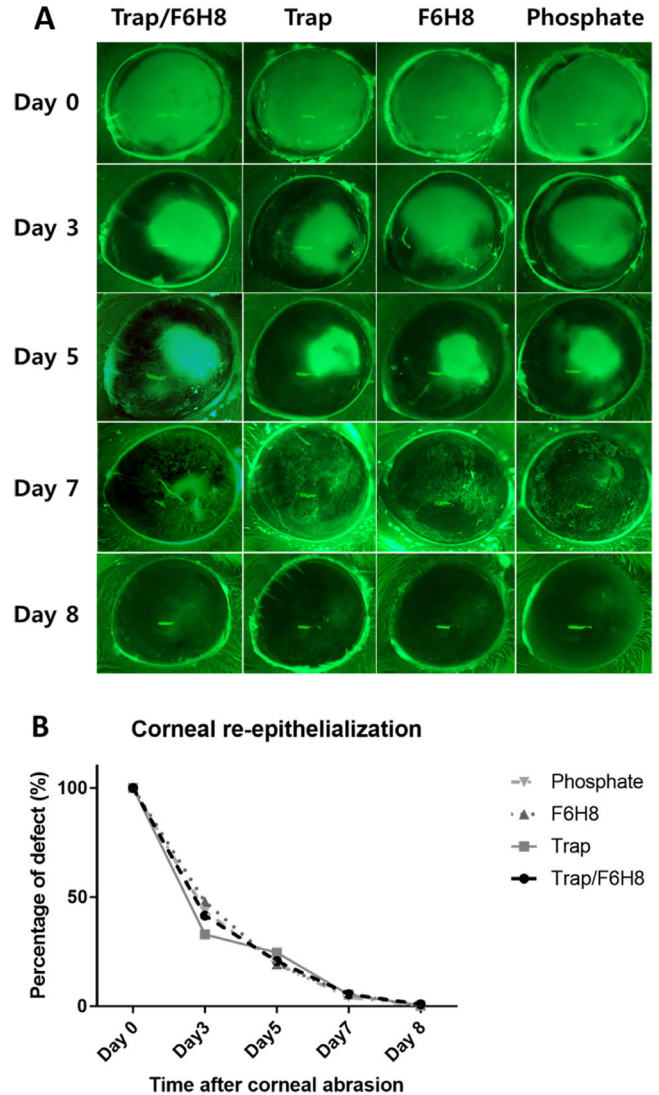, the use of SFAs as drug carriers might, in addition to the effect of the dissolved/suspended drug itself, have a beneficial impact on the ocular surface in terms of superior lubrication and tear film stabilization. In fact, patients with corneal neovascularization often show additional signs and symptoms of dry eye disease, as common etiologies of corneal neovascularization are established risk factors for the development of dry eye disease (e.g. herpetic keratitis [neurotrophic keratopathy] or chemical burns [conjunctival goblet cell loss, limbal stem cell deficiency]).[41–43] Therefore, VEGF Trap$_{R1R2}$ suspended in F6H8 might not only be an effective treatment of corneal neovas-

cularization, but also of co-existing ocular surface disease.

Interestingly, topical application of Trap/F6H8 as well as Trap reduced the number of lymphatic vessel branches and lymphatic vessel end points in the early phase without having a significant overall effect on the corneal area covered by lymphatic vessels. Analysis at later time points, however, showed a significant inhibition of corneal lymphangiogenesis. Thus, blockade of VEGF-A seems to have a faster effect on corneal hemangiogenesis compared to lymphangiogenesis. This finding might support the concept that VEGF-A has indirect effects on corneal lymphangiogenesis, mainly by the recruitment of leukocytes, which in turn are the source of (additional) pro-lymphangiogenic factors.[18] In fact, our current work also shows that treatment with Trap/F6H8 or Trap inhibited early recruitment of inflammatory cells into the cornea after suture placement, which is in line with previously published findings.[18] Thus, one can speculate that blockade of VEGF-A reduces corneal leukocyte infiltration in the early phase, which leads to reduced secretion of pro-lymphangiogenic growth factors. As a result, less lymphangiogenesis can occur during the further course of the vascular response.

In conclusion, our study demonstrates that F6H8 is a potential carrier for VEGF Trap$_{R1R2}$ for the topical treatment of corneal neovascularization. The use of F6H8 as carrier for anti-angiogenic drugs might have an additional positive impact on often co-existing ocular surface disease in patients with corneal neovascularization.

Acknowledgments

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