The Contribution of Macrophages to Inflammatory Corneal Lymphangiogenesis

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Summary

The cornea is physiologically devoid of blood and lymphatic vessels. However, severe inflammation can result in a secondary ingrowth of both vessel types, a process termed “corneal neovascularization”. Macrophages seem to play an important role in this process, although the underlying mechanisms are not well defined. Corneal blood vessels lead to reduced visual acuity when they grow into the optical center, and lymphatic vessels contribute to corneal transplant rejection, dry eye disease, or ocular allergy. In this regard, lymphatic vessels facilitate antigen presenting cell trafficking to the lymph nodes, where accelerated sensitization against allo- or autoantigens occurs. Thus, persistent corneal neovascularization is considered as harmful, and anti(lymph)angiogenic therapy has recently emerged as a novel approach in the treatment of several inflammatory corneal diseases.

One aim of this work was to identify targets for anti(lymph)angiogenic therapy at the cornea and to identify effective anti(lymph)angiogenic compounds, which might be used to treat corneal neovascular diseases. Using a mouse model of sterile corneal inflammation, we found that glucocorticosteroids are strong inhibitors of corneal (lymph)angiogenesis. Glucocorticosteroids suppressed macrophage infiltration into the cornea and inhibited pro-inflammatory cytokine expression in macrophages. Furthermore, we identified insulin receptor substrate-1 (IRS-1) as a mediator of inflammatory corneal (lymph)angiogenesis. IRS-1 was expressed by corneal macrophages, and inhibition of IRS-1 reduced vascular endothelial growth factor (VEGF)-A, VEGF-C and VEGF-D expression in these cells. Consistently, treatment of inflamed corneas with GS-101, an antisense oligonucleotide directed against IRS-1, strongly inhibited inflammatory corneal (lymph)angiogenesis.

Another aim of this study was to analyze whether corneal lymphangiogenesis might also have *beneficial* functions, as studies in extraocular tissues had demonstrated that lymphatic vessels are also important to terminate inflammatory responses. However, studies showing similar functions for corneal lymphatic vessels were missing and the mediators of this putative anti-inflammatory process were unknown. Here, we analyzed the role of Interleukin-10 (IL-10), a primarily anti-inflammatory cytokine, in the regulation of inflammatory corneal lymphangiogenesis. IL-10 was expressed in inflamed corneas by infiltrating macrophages. Furthermore, macrophages treated with IL-10 upregulated pro-lymphangiogenic VEGF-C expression, which is known to induce lymphatic vessel growth. IL-10 deficiency or conditional ablation of IL-10 signaling specifically in myeloid cells lead to reduced inflammatory corneal lymphangiogenesis and prolonged corneal inflammation, whereas treatment with IL-10 promoted lymphangiogenesis and faster egress of macrophages from inflamed corneas. These results collectively indicate that IL-10 indirectly regulates corneal lymphangiogenesis and resolution of corneal inflammation via macrophages, which is the first report of a beneficial function of corneal lymphangiogenesis in (sterile) corneal inflammation.

Taken together, we have identified novel anti(lymph)angiogenic compounds, which mainly affect the contribution of macrophages to inflammatory corneal lymphangiogenesis. Furthermore, we have described a novel anti-inflammatory role for corneal lymphangiogenesis, which is mediated by macrophages. Our work highlights the importance of macrophages for corneal lymphangiogenesis, and might contribute to future immunomodulatory therapeutic strategies promoting corneal repair or preventing disease.

Zusammenfassung

Die Hornhaut enthält physiologisch keine Blut- und Lymphgefäße. Schwere Entzündungen können allerdings zu einem sekundären Einwachsen von Blut- und Lymphgefäßen in die Hornhaut führen. Korneale Blutgefäße reduzieren die Transparenz der Hornhaut, wenn sie in das optische Zentrum einwachsen, und korneale Lymphgefäße tragen zu Abstoßungsreaktionen nach Hornhaut-transplantation, dem trockenen Auge oder der okulären Allergie bei. Hierbei erleichtern Lymphgefäße die Migration von Antigen-präsentierenden Zellen in die Lymphknoten, wo es zu einer Sensibilisierung gegenüber Allo- oder Autoantigenen kommt. Daher werden korneale Blut- und Lymphgefäße in der Regel als nachteilig angesehen, und anti(lymph)angiogene Therapie scheint ein neuer Ansatz bei der Behandlung verschiedener entzündlicher Hornhauterkrankungen zu sein.

Ein Ziel dieser Arbeit war, Zielstrukturen für eine anti(lymph)angiogene Therapie und wirksame Therapeutika zu identifizieren, die zur Therapie von neovaskulären Hornhauterkrankungen genutzt werden könnten. Wir konnten zeigen, dass Glukokortikosteroide potente (Lymph)angiogenese-Inhibitoren sind. Glukokortikosteroide unterdrücken die Rekrutierung von Makrophagen und hemmen die pro-inflammatorische Zytokin-Expression dieser Zellen. Darüber hinaus konnten wir zeigen, dass insulin receptor substrate-1 (IRS-1) eine wichtige Rolle bei der kornealen (Lymph)angiogenese spielt. IRS-1 wird u.a. von Makrophagen exprimiert, und eine Hemmung von IRS-1 reduziert die Expression von vascular endothelial growth factor (VEGF)-A, VEGF-C und VEGF-D in diesen Zellen. Eine Behandlung entzündeter Hornhäute mit GS-101, einem Antisense-Oligonukleotid der gegen IRS-1 gerichtet ist, inhibiert die entzündliche (Lymph)angiogenese an der Hornhaut.

Weiteres Ziel dieser Arbeit war es zu analysieren, ob korneale Lymphgefäße auch *positive* Funktionen haben können, wie es in Arbeiten an extraokulären Geweben bereits gezeigt wurde. Dabei scheinen Lymphgefäße wichtig für das Beenden von Entzündungsreaktionen zu sein. Studien, die ähnliche Funktionen an der Hornhaut zeigen, fehlten bisher und die Mediatoren dieser vermeintlich anti-entzündlichen Funktion waren unbekannt. Wir untersuchten daher die Rolle des anti-inflammatorischen Zytokins Interleukin-10 (IL-10). Wir konnten zeigen, dass IL-10 während einer Entzündungsreaktion durch infiltrierende Makrophagen exprimiert wird, welche nach Stimulation mit IL-10 die Expression von VEGF-C steigern. Das Fehlen von IL-10 oder eine Blockade des IL-10 Signalweges in myeloischen Zellen führt zu einer reduzierten Lymphangiogenese und einer persistierenden Entzündungsreaktion, während eine Stimulation mit IL-10 Lymphangiogenese induziert und zu einem schnelleren Austritt von Makrophagen aus der Hornhaut führt. Somit konnten wir zeigen, dass IL-10 indirekt über Makrophagen die korneale Lymphangiogenese und die Auflösung von Entzündungsprozessen reguliert. Dies ist der erste Nachweis einer positiven Funktion kornealer Lymphgefäßen.

Zusammenfassend haben wir neue anti(lymph)angiogene Therapeutika identifiziert, die Ihren Effekt überwiegend durch Modulation von Makrophagen erzielen. Darüber hinaus haben wir eine neue, anti-entzündliche Rolle für korneale Lymphgefäße aufgezeigt, die durch Makrophagen vermittelt wird. Unsere Arbeit betont die Bedeutung von Makrophagen für die korneale Lymphangiogenese und kann dazu beitragen, künftige immunmodulatorische Therapiestrategien zur Förderung von Reparaturprozessen oder zur Verhütung von Krankheiten an der Hornhaut zu entwickeln.

1. Introduction

1.1. The cornea

The cornea is the outermost part and the major refractive element of the eye. It is physiologically transparent, about 550 µm thick and consists of five layers: 1) a multi-layered squamous non-keratinized epithelium at the outside; 2) the subjacent basement membrane-like Bowman's layer; 3) a middle stromal layer which is sparsely populated with resident tissue cells and mainly consists of collagen fibrils; and the inner layers of 4) Descemet´s membrane and 5) corneal endothelium (Figure 1). The corneal epithelium is covered by the tear film, which contains an aqueous phase secreted by the lacrimal glands, a mucinous phase built by the globlet cells of the conjunctiva, and a lipid phase secreted by the meibomian glands at the lid margin. The tear film and the corneal epithelium form an important barrier to the outside environment and guard the eye from microbial invasion, chemical and toxic damage and foreign bodies, to which the cornea is constantly exposed due to its anatomical position. The corneal stroma, which underlies the epithelium and comprises approximately 80 to 90% of the cornea´s total thickness, shows a highly periodical distribution of its collagen fibrils to minimize light scattering. In addition, continuous dehydration of the stroma by the carbonic anhydrase activity of the corneal endothelial cells results in compactly packed collagen lamellae that ensures transparency.

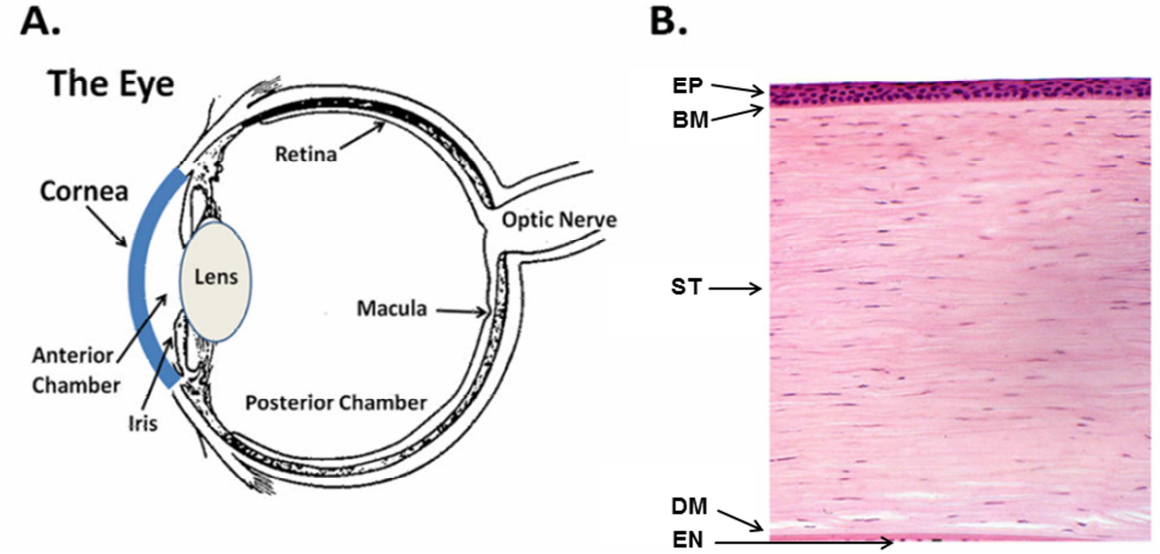

Figure 1: Eye anatomy and corneal histology. A. Schematic diagram of the human eye. B. Histology section of H&E stained human cornea; EP: epithelium, BM: Bowman's layer, ST: stroma, DM: Descemet´s membrane, EN: endothelium (modified from (Leal and Pearlman 2012))

1.2. Corneal (lymph)angiogenic privilege

The cornea is one of the very few tissues of the organism that is devoid of blood and lymphatic vessels (Cursiefen 2007). This absence of blood and lymphatic vessels, termed “corneal (lymph)angiogenic privilege” is not a passive condition but is rather actively maintained. Recently, several mechanisms that contribute to this privilege have been identified. In this regard, the corneal epithelium plays a pivotal role in maintaining corneal avascularity, as it expresses soluble forms of the vascular endothelial growth factor (VEGF) receptors (sVEGFR-1, sVEGFR-2, sVEGFR-3), which function as decoy receptors for the key (lymph)angiogenic growth factors VEGF-A, VEGF-C and VEGF-D (Albuquerque et al. 2009; Ambati et al. 2006; Singh et al. 2013). In addition, the corneal epithelium expresses (non-vascular) membrane-bound VEGFR-3 (mVEGFR-3), which is also able to trap VEGF-C and VEGF-D (Cursiefen et al. 2006). Further potent anti(lymph)angiogenic molecules expressed in the cornea are angiostatin, endostatin, pigment epithelium-

derived factor, thrombospondin-1, and thrombospondin-2, which all exhibit multiple inhibitory functions such as direct inhibition of vascular endothelial cell migration and proliferation as well as indirect interference with growth factor mobilization and binding (Armstrong and Bornstein 2003; Cursiefen et al. 2011; Cursiefen et al. 2004; Lin et al. 2001). Especially thrombospondin-1 is a crucial molecule for corneal alymphaticity, as it has been shown to inhibit the expression of VEGF-C by corneal inflammatory cells. Accordingly, thrombospondin-1 deficient mice show increased corneal VEGF-C expression and also higher inflammatory cell numbers, which result in spontaneous and isolated ingrowth of lymphatic vessels into the cornea (Cursiefen et al. 2011). Another antiangiogenic mechanism of the cornea is the expression of the inhibitory PAS domain protein (IPAS), which negatively regulates hypoxia-induced upregulation of VEGF and thereby maintains corneal avascularity even under hypoxic conditions (Makino et al. 2001).

Despite of its avascular and alymphatic nature, the cornea also relies on blood components to remain healthy. Delivery of nutrients and subsequent clearance of metabolites are carried out by the tear film from the corneal surface, the aqueous humour from the anterior chamber and at the limbus from the lateral margin. The limbus is a transition zone where the cornea fades into the opaque and vascularized sclera and overlying conjunctiva. In contrast to the cornea, the limbus is physiologically vascularized: limbal blood vessel arterioles originate from the anterior ciliary arteries, sprout towards the corneal border and form small pericorneal arcades. The lymphatic vasculature of the limbus also consists of a ring shaped network which is connected to the conjunctival lymphatic vessels. Limbal lymphatic vessels do not form arcades but rather consist of a main circumferential

lymphatic vessel with blind ending extensions directed towards the cornea (Figure 2).

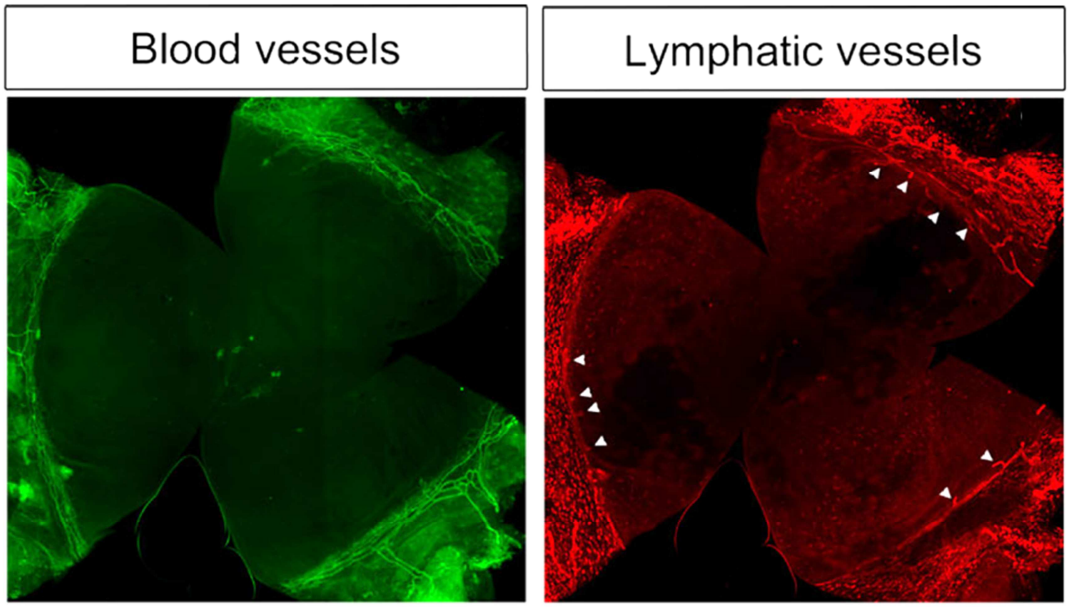

Figure 2: Vascular anatomy of the cornea. Whole mounts of healthy murine corneas stained with CD31 (blood vessels; left panel) or LYVE-1 (lymphatic vessels, right panel; arrowheads indicate centrally oriented vascular extensions from the main limbal lymphatic vessel). Blood and lymphatic vessels terminate at the limbal border (modified from (Hos et al. 2013)).

1.3. Pathological corneal (lymph)angiogenesis

Corneal avascularity and alymphaticity are not invulnerable. Whereas minor inflammatory vascular stimuli are buffered by the corneal (lymph)angiogenic privilege and do not induce an angiogenic response, severe tissue damage and inflammation can result in a strong upregulation of pro(lymph)angiogenic growth factors, which might “overwhelm” the cornea’s anti(lymph)angiogenic mechanisms and lead to an ingrowth of blood and clinically invisible lymphatic vessels from the limbus into the cornea (corneal [lymph]angiogenesis) (Cursiefen 2007). Diseases that can result in corneal (lymph)angiogenesis include infectious (e.g. bacterial, viral, or fungal keratitis), inflammatory (e.g. ocular pemphigoid, Lyell-Syndrome, Stevens-Johnson Syndrome, Graft-versus-Host Disease, corneal graft rejection),

hypoxic (e.g. extended wear of contact lenses), and traumatic/toxic diseases (e.g. chemical or physical burns). Diseases that lead to a loss of limbal barrier function (e.g. limbal stem cell deficiency) are also frequently accompanied by corneal (lymph)angiogenesis. Blood and lymphatic vessels mostly grow in parallel into the cornea and it seems that the degree of corneal hemangiogenesis generally correlates with the degree of corneal lymphangiogenesis (Cursiefen et al. 2006; Cursiefen et al. 2002).

Corneal (lymph)angiogenesis after tissue damage possibly has several important functions including supply of components of cellular and humoral immunity to combat infections, supply of additive oxygen and growth factors to promote the healing response, and drainage and removal of debris and cells from the injured site. However, this process might also cause loss of transparency in case of blood vessel growth into the optical zone, which can lead to light scattering or obscuration. Furthermore, also secondary effects such as fluid and lipid deposition through immature capillaries and exuberant influx of inflammatory cells might contribute to corneal edema, loss of corneal compactness and opacification (Cursiefen 2007). Therefore, after the repair response is completed and barrier function has been achieved, corneal blood vessels must resolve rapidly to restore corneal transparency and thereby functionality. However, under certain pathologic conditions and chronic inflammatory diseases, corneal blood vessels regress only very slowly or may even persist and permanently impair vision (Figure 3). Whereas it is well accepted that persisting pathological corneal blood vessels contribute to loss of corneal transparency, the contribution of clinically invisible *lymphatic* vessels to corneal diseases was less apparent. Recently, however, several experimental

studies provided evidence for the contribution of corneal lymphatic vessels to ocular pathologies, such as transplant rejection and dry eye disease (Dietrich et al. 2010; Goyal et al. 2010). To date, corneal lymphangiogenesis is therefore considered as pathological and undesirable. However, it is well known that in extraocular tissues, lymphatic vessels exert important physiological functions during the inflammatory response. Lymphatic vessels regulate tissue pressure and allow the drainage of debris and egress of inflammatory cells from the injured site (Oliver and Detmar 2002). In particular, it has been shown that lymphatic vessels seem to be important for the termination of inflammatory responses and recent studies show that the inhibition of lymphangiogenesis might lead to chronic inflammation and edema, whereas the specific activation of lymphatic vessels can ameliorate these conditions (Huggenberger et al. 2011; Huggenberger et al. 2010). However, studies demonstrating similar beneficial functions for lymphatic vessels at the cornea are still missing.

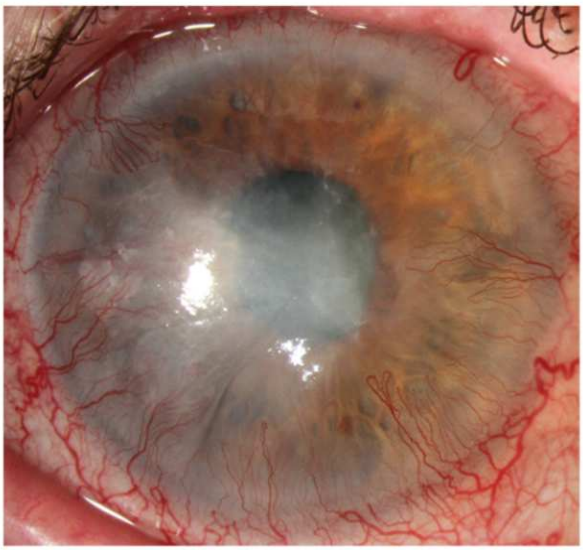

Figure 3: Corneal neovascularization. Clinical picture of a patient with pathological corneal blood vessels secondary to recurrent herpetic ulcerative keratitis. Blood vessels originate from the limbus and grow centrally towards the cornea. Note secondary central corneal scar (from (Hos et al. 2016)).

1.4. Corneal (lymph)angiogenesis as a risk factor for corneal graft rejection

Corneal transplantation is the oldest, most common and the most successful form of (tissue) transplantation (Streilein et al. 1999). The reason for this extraordinary success is that the cornea is considered to be an immune-privileged anatomical structure, as it: 1) contains only very low numbers of MHC class II positive antigen presenting cells (APCs) in the corneal centre; 2) shows generally reduced expression of MHC class I; and 3) expresses Fas ligand (CD95L) and programmed death ligand 1 (PD-L1), which inhibit T cell responses. Another important mechanism responsible for the corneal immune privilege is the phenomenon of anterior chamber-associated immune deviation (ACAID), which causes antigen-specific systemic immune tolerance characterized by downregulation of delayed-type hypersensitivity and cytotoxic T cells responses (Niederkorn 2010; Niederkorn 1999; Streilein et al. 1999). Interestingly, it has been shown that the corneal immune privilege depends, at least partially, on its intact (lymph)angiogenic privilege (Cursiefen 2007).

When corneal transplantations are performed because of non-inflammatory and non-vascular diseases with intact (lymph)angiogenic and immune privilege, e.g. because of corneal (endothelial) dystrophies, more than 75% of corneal grafts survive for longer than 5 years, even without previous HLA-matching and without any systemic immunosuppressive therapy (Williams and Coster 2007). However, when severe inflammatory processes have overcome the cornea´s (lymph)angiogenic and immune privilege and donor corneas are grafted into prevascularized recipients, transplant survival rates significantly decrease, even under aggressive systemic immunosuppression. Therefore, vascularization of the

cornea prior to transplantation is considered as one of the most important risk factors for immunological graft rejection (Dana and Streilein 1996; Sano et al. 1995). Preclinical evidence suggests that the reason for decreased graft survival rates in prevascularized corneas is due to an enhanced facilitation of allogenic immune responses: via preexisting corneal lymphatic vessels, donor and antigen-loaded recipient APCs have direct and immediate access to the regional lymph nodes, where increased and accelerated allosensitization occurs (Cursiefen et al. 2003; Hos and Cursiefen 2014). Subsequently, via preexisting corneal blood vessels, immune effector cells, such as alloreactive T cells, can easily reach and subsequently reject the graft (Figure 4).

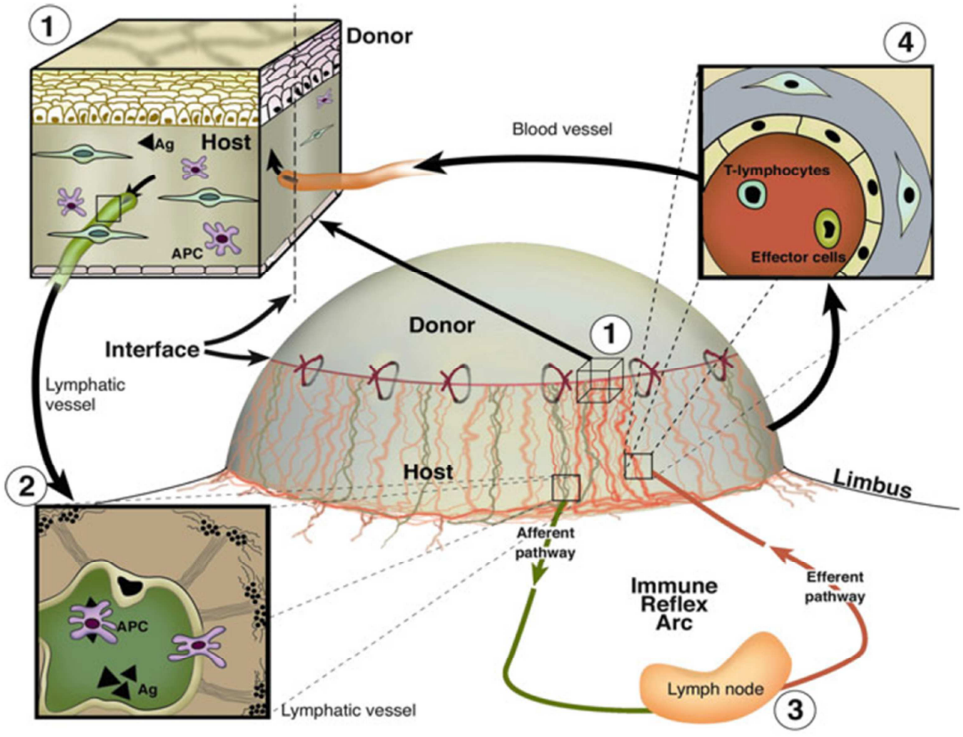

Figure 4: The role of blood and lymphatic vessels in corneal transplant rejection. Schematic drawing illustrating the role of blood and lymphatic vessels in corneal transplantations performed in pathologically prevascularized recipients. (1) Magnification of the host-graft interface: blood (red) and lymphatic vessels (green) reach the host-graft interface. Antigen-presenting cells (APCs) and antigen (Ag) can leave the cornea via corneal lymphatic vessels (2) and reach the regional lymph nodes (3); after stimulation of alloreactive T cells, these and other effector cells can reach the graft via corneal blood vessels (4) (from (Cursiefen et al. 2003)

As persistent pathological blood and lymphatic vessels play such an important role in the development of corneal graft rejection, several groups have analyzed the impact of a pharmacological inhibition of corneal (lymph)angiogenesis in the context of corneal transplantation. Importantly, blockade of corneal hem- and lymphangiogenesis significantly promoted corneal graft survival in the experimental setting (Cursiefen et al. 2004; Hos et al. 2008). Moreover, using specific pharmacological drugs or adenoviral vectors that modulate lymphangiogenesis without affecting hemangiogenesis, it was demonstrated that lymphatic vessels - which ease the connection between the graft and the secondary lymphatic organs - play a superior role in the mediation of graft rejection when compared to blood vessels (Albuquerque et al. 2009; Dietrich et al. 2010) (Figure 5). Thus, antilymphangiogenic therapy at the cornea has recently emerged as a novel therapeutic concept to reduce corneal graft rejection, and recent efforts aim to identify effective anti(lymph)angiogenic compounds which might potentially be used in the clinic in the near future (Bock et al. 2013).

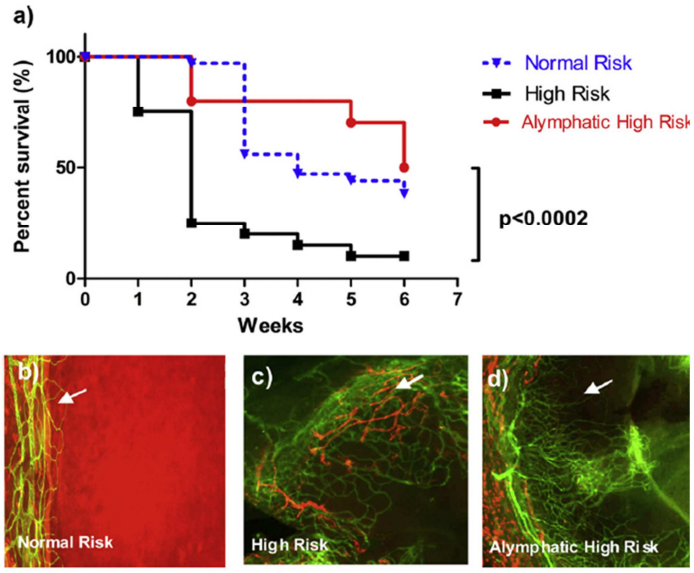

Figure 5: Lymphatic vessels define the high risk status of transplants in prevascularized corneal hosts. (a) Kaplan-Meier survival curve showing that absence of lymphatic vessels in the recipient cornea prior to transplantation improves graft survival in the experimental setting;(b to d) corneal whole mounts stained with CD31 (blood vessel; green) and LYVE-1 (lymphatic vessels, red) in different recipient beds (from (Hos et al. 2014)).

1.5. The role of corneal lymphangiogenesis in dry eye disease

Dry eye disease is one of the most frequent diseases in ophthalmology. It is generally accepted that it is not only a condition with reduced tear quantity, but rather a complex, multifactorial disorder of the ocular surface where chronic inflammation plays an important role and where influx and activation of inflammatory cells results in disturbed tear production, abnormal tear composition and subsequent damage of the ocular surface (Barabino et al. 2012; De Paiva et al. 2009). Recent evidence suggests that also the adaptive immune system is involved in maintaining ocular surface inflammation in dry eye disease (Stern et al. 2013; Stevenson et al. 2012). Interestingly, it has been shown in several mouse models that in this disease, an isolated ingrowth of lymphatic but not blood vessels into the cornea can be observed (Cursiefen et al. 2011; Goyal et al. 2010). These corneal lymphatic vessels seem to serve as conduits that enable easier autoantigen-transport and accelerated trafficking of activated APCs from to the ocular surface to the lymph nodes (Goyal et al. 2010; Stevenson et al. 2012). Furthermore, it has been shown that inhibition of corneal lymphangiogenesis significantly improves the clinical course of dry eye, at least in the experimental setting (Goyal et al. 2012). Therefore, antilymphangiogenic therapy might offer a promising approach not only to prevent graft rejection after corneal transplantation, but also to treat dry eye disease.

1.6. The contribution of macrophages to corneal (lymph)angiogenesis

Macrophages are derived from blood monocytes, which originate in the bone marrow and are released into blood circulation. Thereafter, pro-inflammatory stimuli

can cause the recruitment of monocytes to injured peripheral sites, where differentiation into (inflammatory) macrophages occurs. Recent evidence suggests that there is also another distinct population of macrophages, which seem to develop independently from the bone marrow but rather from progenitor cells generated in the yolk sac (Schulz et al. 2012). These macrophages represent the resident tissue macrophage population and are important for maintaining tussue homeostasis during “steady-state”. However, to which extent this also accounts for corneal tissue macrophages is so far undetermined.

Substantial preclinical evidence indicates that macrophages are essential mediators of corneal (lymph)angiogenesis after injury (Chung et al. 2009; Cursiefen et al. 2004). It is known that macrophages are able to secrete VEGF-A, VEGF-C und VEGF-D and thereby induce vascular endothelial cell proliferation and migration (Cursiefen et al. 2004). In addition, macrophages also express the respective receptors (VEGFR-1 and VEGFR-3), which mediate chemotactic effects and thereby amplify the inflammatory (lymph)angiogenic response (Cursiefen et al. 2004). Furthermore, under certain, so far still poorly understood conditions, macrophages are able to express lymphendothelial markers and integrate into newly formed corneal lymphatic vessels or even generate primitive lymphatic vessel-like structures de novo (Maruyama et al. 2005). Moreover, macrophages are frequently localized in close proximity to already formed blood and lymphatic vessels, suggesting a continuing interaction with these (Maruyama et al. 2005). The crucial role of macrophages in mediating corneal (lymph)angiogenesis is further supported by the fact that macrophage depletion, e.g., by clodronate liposomes,

almost completely prevents inflammatory corneal (lymph)angiogenesis (Cursiefen et al. 2004).

Macrophages are a heterogeneous, highly plastic cell population and various functional phenotypes have been described (Gordon and Taylor 2005; Sica and Mantovani 2012). One current conceptual model classifies macrophages into at least two subpopulations (paradigm of M1/M2 polarization). In this model, “classically activated” (also called M1-polarized) macrophages are considered to exert pro-inflammatory activities, promote type I immune responses and are involved in the eradication of invading microorganisms. In contrast, “alternatively activated” (also called M2-polarized) macrophages, which are hyporesponsive to pro-inflammatory stimuli, are mainly involved in debris scavenging, tissue remodeling and the resolution of inflammatory responses (Gordon and Martinez 2010). Classical macrophage activation is mediated by pro-inflammatory stimuli, such as IFN-γ or TNF-α, followed by a microbial trigger (e.g. lipopolysaccharide). Mediators of alternative macrophage activation are IL-4 and IL-13, or IL-10 (Gordon and Taylor 2005). By now, however, it is becoming clear that the paradigm of M1/M2 polarization is an oversimplification and reflects two extremes of macrophage polarization, and that in tissues a broad spectrum of activation states exists in parallel (Martinez and Gordon 2014). Although macrophage biology is an extensively studied research field, there are still numerous unaddressed questions. In the context of corneal inflammation for instance, it is so far unknown whether specific macrophage subpopulations occur and which macrophage subpopulations mediate corneal (lymph)angiogenesis. It is also unclear whether macrophages exert

a different (lymph)angiogenic potential during the various stages of corneal inflammation.

Taken together, macrophages are known to be importantly involved in inflammatory corneal (lymph)angiogenesis. However, the specific mechanisms are not yet identified. Thus, more detailed analysis of corneal macrophage activation and polarization is required, before macrophages can be considered as a potential tool to control corneal physiology or disease. On the one hand, inhibition of pro-(lymph)angiogenic macrophage subsets would be an interesting therapeutic option to modulate corneal (lymph)angiogenesis, e.g. in the context of cornea transplantation or dry eye disease. On the other hand, if corneal lymphangiogenesis and pro-lymphangiogenic macrophages would be involved in physiological wound healing responses in the cornea, specific *activation* of these would be of great interest to promote corneal repair.

1.7. Aims of the thesis

Persistent corneal lymphatic vessels contribute to the development of detrimental corneal pathologies, such as corneal graft rejection or dry eye disease. Antilymphangiogenic therapy at the cornea has emerged as a promising approach to treat these inflammatory diseases. Therefore, this study aimed to identify effective antilymphangiogenic compounds, which might potentially be used to treat patients at risk for corneal graft rejection or with dry eye disease. In addition, this work aimed to find novel potential targets for antilymphangiogenic therapy at the cornea.

In the first part of this thesis, we analyzed the impact of glucocorticosteroids on inflammatory corneal lymphangiogenesis and investigated the potential

underlying mechanisms. This was of particular interest, as glucocorticosteroids are widely used as the standard anti-inflammatory treatment at the cornea and have also been shown to reduce the risk of corneal graft rejection and to ameliorate dry eye disease (Nguyen et al. 2007). However, little was known about the ability of these drugs to suppress corneal lymphangiogenesis.

In the second part of this work, we analyzed whether inflammatory corneal lymphangiogenesis is regulated by insulin receptor substrate-1 (IRS-1). IRS-1 is a cytosolic scaffolding protein that interacts with the VEGFR complex, and it was recently shown that IRS-1 plays a role in blood vessel development (Andrieu-Soler et al. 2005; Miele et al. 2000). However, it was unclear whether IRS-1 is also involved in lymphatic vessel development and whether IRS-1 is a potential target to treat corneal lymphangiogenesis.

In contrast to the role of corneal lymphatic vessels under pathological conditions, a *physiological* function for corneal lymphatic vessels has not been described so far. Studies in extraocular tissues have demonstrated that lymphangiogenesis might also be important to terminate inflammatory responses (Huggenberger et al. 2011). However, studies showing similar beneficial functions for corneal lymphatic vessels are missing and the mediators of this putative anti-inflammatory lymphangiogenesis are unknown.

Therefore, in the third part of this thesis, we analyzed the role of Interleukin-10 (IL-10), a primarily anti-inflammatory cytokine, in the regulation of inflammatory corneal lymphangiogenesis. We determined the impact of IL-10 on macrophages, inflammatory corneal lymphangiogenesis and the resolution of corneal inflammation.

2. Results

2.1. Suppression of inflammatory corneal lymphangiogenesis by application of topical corticosteroids.

Deniz Hos, Daniel R. Saban, Felix Bock, Birgit Regenfuss, Jasmine Onderka, Sharmila Masli, Claus Cursiefen

Objectives: To analyze whether topical application of corticosteroids inhibits inflammatory corneal lymphangiogenesis and to study the potential underlying antilymphangiogenic mechanisms.

Methods: Inflammatory corneal neovascularization was induced by suture placement, and the corneas were then treated with topical fluorometholone, prednisolone acetate, or dexamethasone sodium phosphate. After 1 week, the corneas were stained with lymphatic vessel endothelial hyaluronan receptor 1 for detection of pathological corneal lymphangiogenesis. The effect of these corticosteroids on macrophage recruitment was assessed via fluorescence-activated cell sorting analysis. The effect of these corticosteroids on proinflammatory cytokine expression by peritoneal exudate cells was tested via real-time polymerase chain reaction. Furthermore, the effect of steroid treatment on the proliferation of lymphatic endothelial cells was assessed via enzyme-linked immunosorbent assay.

Results: Treatment with corticosteroids resulted in a significant reduction of inflammatory corneal lymphangiogenesis. The antilymphangiogenic effect of fluorometholone was significantly weaker than that of prednisolone and dexamethasone. Corneal macrophage recruitment was also significantly inhibited by

the application of topical steroids. Treatment of peritoneal exudate cells with corticosteroids led to a significant downregulation of the RNA expression levels of tumor necrosis factor and interleukin 1β. Additionally, proliferation of lymphatic endothelial cells was also inhibited.

Conclusions: Corticosteroids are strong inhibitors of inflammatory corneal lymphangiogenesis, with significant differences between various corticosteroids in terms of their antilymphangiogenic potency. The main mechanism of the antilymphangiogenic effect seems to be through the suppression of macrophage infiltration, proinflammatory cytokine expression, and direct inhibition of proliferation of lymphatic endothelial cells.

Clinical relevance: Steroids block corneal lymphangiogenesis, the main risk factor for immune rejections after corneal transplantation. The different antilymphangiogenic potency of these drugs should be taken into account when using steroids in clinical practice (e.g., after keratoplasty).

Own contribution to publication 1:

I performed all in vivo inflammatory corneal neovascularization assays, treated all animals with the respective drugs and subsequently performed all whole mount immunohistochemical stainings and vessel analyses (Figure 1). I also performed the in vivo experiments and treatments for the FACS analysis and assisted in FACS data acquisition and interpretation (Figure 2). I further isolated the peritoneal macrophages, carried out all in vitro treatments and performed and analyzed the quantitative real-time PCRs (Figure 3). In addition, I performed and analyzed the lymphatic endothelial proliferation assays (Figure 4). All images

illustrating my experimental data were generated by me. I wrote the manuscript and handled the revision phase of this project.

Contribution of co-authors to publication 1:

Dr. Daniel R. Saban established, performed and analyzed the FACS experiments (Figure 2). Dr. Felix Bock and Dr. Birgit Regenfuss provided help in analyzing the in vivo angiogenesis data (Figure 1), provided valuable general suggestions and critically read the manuscript. Jasmine Onderka assisted and provided support in lymphatic endothelial culture and in vitro proliferation assays (Figure 4). Dr. Sharmila Masli provided help in isolation and culture of peritoneal macrophages (Figure 3). Prof. Claus Cursiefen conceptualized and coordinated the project and wrote the manuscript.

2.2. Blockade of insulin receptor substrate-1 inhibits corneal lymphangiogenesis.

Deniz Hos, Birgit Regenfuss, Felix Bock, Jasmine Onderka, Claus Cursiefen

Purpose: To analyze whether insulin receptor substrate (IRS-1) is involved in lymphatic vessel development and whether IRS-1 blockade can inhibit lymphangiogenesis in vivo.

Methods: The impact of IRS-1 blockade by GS-101 (Aganirsen), an antisense oligonucleotide against IRS-1, on lymphatic endothelial cell (LEC) proliferation was assessed by ELISA. Furthermore, the effect of IRS-1 blockade on prolymphangiogenic growth factor expression by LECs and macrophages (peritoneal exudate cells) was tested by real-time PCR. The mouse model of inflammatory corneal neovascularization was used to analyze the effect of IRS-1 blockade in vivo: after corneal suture placement, mice were treated with GS-101 eye drops (twice daily afterwards for 1 week, 5 µL per drop; 50, 100, or 200 µM). Afterward, corneal wholemounts were prepared and stained for blood and lymphatic vessels.

Results: Blockade of IRS-1 by GS-101 inhibited LEC proliferation dose dependently. GS-101 led to decreased VEGF-A expression levels in LECs, whereas VEGF-C, VEGF-D, and VEGFR3 showed no significant change. In macrophages, VEGF-A expression levels were also inhibited by IRS-1 blockade. Additionally, GS-101 strongly inhibited macrophage-derived VEGF-C, VEGF-D, and VEGFR3 expression. In vivo, corneal hemangiogenesis was significantly inhibited when used at a concentration of 200 µM (by 17%; $P < 0.01$). Corneal lymphangiogenesis was significantly inhibited when used at a dose of 100 µM (by 21%; $P < 0.01$), and the

highest used dose (200 µM) showed an even stronger inhibition (by 28%; $P < 0.001$).

Conclusions: Blockade of IRS-1 inhibits not only hemangiogenesis but also lymphangiogenesis. To the authors' knowledge, this is the first evidence that IRS-1 is involved in the molecular pathway leading to lymphangiogenesis.

Own contribution to publication 2:

I performed and analyzed all lymphatic endothelial proliferation assays (Figure 1). Furthermore, I performed and analyzed all real-time PCRs with lymphatic endothelial cells (Figure 2). I also isolated the peritoneal macrophages, carried out all in vitro treatments and performed and analyzed the real-time PCRs (Figure 3). The in vivo inflammatory corneal neovascularization assays, all treatments and whole mount immunohistochemical stainings with subsequent vessel analyses were carried out by me (Figure 4). In addition, I performed the corneal macrophage stainings (Figure 5). All images illustrating my experimental data were generated by me. I wrote the manuscript and handled the revision phase of this project.

Contribution of co-authors to publication 2:

Dr. Birgit Regenfuss provided help in establishing the real-time PCR protocols (Figure 2 and 3). In addition, Dr. Birgit Regenfuss and Dr. Felix Bock supported me in analyzing the in vivo angiogenesis data (Figure 4), provided general suggestions and corrected the manuscript. Jasmine Onderka assisted in lymphatic endothelial culture, in vitro proliferation assays (Figure 1) and histological stainings (Figure 5). Prof. Claus Cursiefen conceptualized and coordinated the project.

2.3. IL-10 Indirectly Regulates Corneal Lymphangiogenesis and Resolution of Inflammation via Macrophages.

Deniz Hos, Franziska Bucher, Birgit Regenfuss, Marie-Luise Dreisow, Felix Bock, Ludwig M. Heindl, Sabine A. Eming, Claus Cursiefen

The role of IL-10, a primarily anti-inflammatory cytokine, in the regulation of inflammatory lymphangiogenesis is undetermined. Herein, we show that IL-10 modulates corneal lymphangiogenesis and resolution of inflammation. IL-10 was not expressed in healthy corneas but was up-regulated in inflamed corneas by infiltrating macrophages. Macrophages up-regulated the expression of prolymphangiogenic vascular endothelial growth factor-C upon stimulation with IL-10. Consistently, corneal inflammation resulted in reduced expression of vascular endothelial growth factor-C and decreased corneal lymphangiogenesis in IL-10-deficient mice (IL-10(-/-)). The effect of IL-10 on lymphangiogenesis was indirect via macrophages, because IL-10 did not directly affect lymphatic endothelial cells. The expression of proinflammatory cytokines and the numbers of infiltrating macrophages increased and remained elevated in inflamed corneas of IL-10(-/-) mice, indicating that IL-10 deficiency led to more severe and prolonged inflammation. The corneal phenotype of IL-10 deficient mice was mimicked in mice with conditional deletion of Stat3 in myeloid cells (lysozyme M Cre mice Stat3(fl/fl) mice), corroborating the critical role of macrophages in the regulation of lymphangiogenesis. Furthermore, local treatment with IL-10 promoted lymphangiogenesis and faster egress of macrophages from inflamed corneas. Taken together, we demonstrate that IL-10 indirectly regulates inflammatory corneal

lymphangiogenesis via macrophages. Reduced lymphangiogenesis in IL-10(-/-) and lysozyme M Cre Stat3(fl/fl) mice is associated with more severe inflammatory responses, whereas IL-10 treatment results in faster resolution of inflammation. IL-10 might be used therapeutically to terminate pathological inflammation.

Own contribution to publication 3:

I conceptualized, performed and analyzed all experiments. In particular, I performed all real-time PCR experiments (Figure 1A, Figure 2, Figure 4A and 4B, Figure 5A and 5B, Figure 6A and 6B, Figure S1B and S1C). I performed the immunhistochemical stainings on cryosectioned mouse corneas (Figure 1B; Figure 3). I performed the lymphatic endothelial proliferation assay (Figure S1A). Protein isolation of inflamed murine corneas and the subsequent VEGF-C protein ELISA was carried out by me (Figure 4C). In addition, I performed the subconjunctival injections of recombinant IL-10 (Figure 7). Furthermore, the in vivo angiogenesis assays and whole mount immunohistochemical stainings with subsequent cell and vessel analyses were carried out by me (Figure 4D to 4I, Figure 5C to 5I, Figure 6C to 6H, Figure 7). All images illustrating my experimental data were generated by me. I wrote the manuscript and handled the revision phase of this project.

Contribution of co-authors to publication 3:

Dr. Franziska Bucher helped in performing parts of the in vivo assays (Figure 4D to 4H, Figure 5C to 5H) and critically read the manuscript. Dr. Birgit Regenfuss provided help in establishing parts of the real-time PCR protocols (Figure 3). In addition, Dr. Birgit Regenfuss analyzed parts of the in vivo angiogenesis data (Figure 4D to 4I), provided valuable general suggestions and critically read the

manuscript. Marie-Luise Dreisow helped in mice genotyping and cryosectioned the corneas for immunhistochemical stainings (Figure 1B, Figure 3). Dr. Felix Bock analyzed parts of the in vivo angiogenesis data (Figure 4D to 4I), provided valuable general suggestions and critically read the manuscript. Prof. Ludwig M. Heindl provided suggestions and critically read the manuscript. Prof. Sabine A. Eming provided the Lysozyme M Cre and floxed Stat3 mice (Figure 6), provided valuable general suggestions and critically read the manuscript. Prof. Claus Cursiefen conceptualized and coordinated the project and wrote the manuscript.

3. Discussion

3.1. Main findings of the three presented studies in summary

1. Glucocorticosteroids are not only inhibitors of inflammatory corneal hem-, but also lymphangiogenesis. Glucocorticosteroids strongly suppress macrophage infiltration into the inflamed cornea and inhibit the expression of proinflammatory cytokines in macrophages. In addition, glucocorticosteroids also directly reduce lymphatic endothelial cell proliferation.

2. Corneal macrophages express insulin receptor substrate -1 (IRS-1), and inhibition of IRS-1 reduces macrophage-derived expression of pro(lymph)angiogenic VEGF-A, VEGF-C and VEGF-D. Topical application of GS-101 (Aganirsen), an antisense oligonucleotide directed against IRS-1, strongly inhibits inflammatory corneal hem- and lymphangiogenesis.

3. IL-10 is expressed in inflamed corneas by infiltrating macrophages. Stimulation of macrophages with IL-10 upregulates the expression of pro-lymphangiogenic VEGF-C. IL-10 deficiency results in less corneal lymphangiogenesis and prolonged corneal inflammation. Local treatment with IL-10 promotes corneal lymphangiogenesis and faster egress of macrophages from inflamed corneas, leading to the resolution of inflammation.

3.2. Novel anti-(lymph)angiogenic treatment strategies for corneal neovascular diseases

A variety of clinical indications exist for anti(lymph)angiogenic treatment at the cornea, e.g., to reduce sight-threatening neovascularization after inflammation, to improve graft survival after corneal transplantation or to treat dry eye disease (Bock et al. 2013).To date, glucocorticosteroid therapy is the standard anti-inflammatory treatment for these diseases and glucocorticosteroids have been shown to reduce the risk of corneal graft rejection and to ameliorate dry eye disease (Marsh and Pflugfelder 1999; Nguyen et al. 2007). This may largely be attributable to the fact that glucocorticosteroids are very potent anti-inflammatory substances. However, whether these drugs are also able to suppress corneal lymphangiogenesis, which crucially contributes to corneal graft rejection and dry eye disease, was unknown. We demonstrated that glucocorticosteroids are strong inhibitors of inflammatory corneal hem- and lymphangiogenesis in vivo. The fact that glucocorticosteroids are also able to inhibit inflammatory corneal lymphangiogenesis may lead one to conclude that both the anti-inflammatory effects and the anti-lymphangiogenic properties contribute to the beneficial effects of these substances in the treatment of corneal graft rejection or dry eye disease.

Corneal flow cytometry analyses revealed that glucocorticosteroids strongly suppress macrophage infiltration into the inflamed cornea. Furthermore, glucocorticosteroids also inhibit the expression of pro-inflammatory cytokines, such as TNF-α and IL-1β in macrophages. We tested several glucocorticosteroids, namely fluorometholone, prednisolone and dexamethasone, and observed that the anti-inflammatory effects correlated with the anti-lymphangiogenic effects. This

supports the fact that macrophages are very important mediators of inflammatory corneal lymphangiogenesis. However, our work does not fully allow the conclusion that the impact of glucocorticosteroids on corneal lymphangiogenesis is solely mediated via the modulation of macrophages, as we demonstrated that glucocorticosteroids also directly reduce lymphatic endothelial cell proliferation. Nevertheless, the strong correlation of the anti-inflammatory and anti-lymphangiogenic effects of glucocorticosteroids suggests a very close interrelation between corneal inflammation, macrophages and (lymph)angiogenesis.

The use of glucocorticosteroids in the management of neovascular corneal diseases remains controversial because of the adverse effects associated with this type of therapy. Although glucocorticosteroids are very potent and useful compounds, the (prolonged) use of these drugs might lead to delayed epithelial wound healing, elevated intraocular pressure, cataract, or increased risk of infections (Becker 1964). Furthermore, although glucocorticosteroids suppress the formation of new vessels in progressive corneal neovascular diseases, clinical experience shows that these drugs are less effective in regressing already present, mature vessels. Furthermore, especially in highly inflamed settings, glucocorticosteroids are not sufficient to fully block corneal neovascularization, even when used at high dosage (Cursiefen et al. 2001). Thus, there is still need for alternative, more specific anti(lymph)angiogenic therapeutic approaches at the cornea.

Recently, several specific angiogenesis inhibitors have been approved by the US Food and Drug Administration for the treatment of pathologic neovascularization at the posterior part of the eye. Ranibizumab (Lucentis), an antibody fragment

directed against VEGF-A, and Aflibercept (Eylea), which targets VEGF-A and placenta growth factor (PlGF), are both approved for the treatment of age related macular degeneration, diabetic macular edema, and macular edema following retinal vein occlusion. Bevacizumab (Avastin), a humanized antibody directed against VEGF-A, is approved for the treatment of several cancer entities and is also widely used off-label to treat vaso-proliferative retinopathies. In contrast to the posterior part of the eye, however, there is no specific angiogenesis inhibitor, which is used to treat neovascularization at the anterior segment of the eye. Therefore, recent efforts try to identify novel targets for anti(lymph)angiogenic therapy at the cornea and test potential (lymph)angiogenesis inhibitors in preclinical models. One promising candidate is insulin receptor substrate-1 (IRS-1), a cytosolic adapter protein that has been shown to interact with the VEGF-receptor complex (Miele et al. 2000). Although no study had analyzed the contribution of IRS-1 to lymphatic vessel growth, it was previously shown that hypoxia regulates IRS-1 expression in endothelial cells and that hypoxic retinal blood vessel growth is reduced in IRS-1 deficient mice (Jiang et al. 2003). Furthermore, it was demonstrated that IRS-1 is also expressed in the cornea, pointing to a potential role of IRS-1 signaling also in corneal neovascularization (Andrieu-Soler et al. 2005). Here, we demonstrate that IRS-1 is expressed by corneal macrophages, and that treatment with GS-101 (Aganirsen, Gene-Signal), an antisense oligonucleotide that blocks the expression of IRS-1, leads to reduced VEGF-A, VEGF-C and VEGF-D levels in these cells. Consistently, topical application of Aganirsen eye drops significantly reduced inflammatory corneal hem- and lymphangiogenesis in treated mice. The anti-(lymph)angiogenic polarization of macrophages by Aganirsen and the fact that it

was previously shown that this compound also decreases the number of macrophage recruitment to inflamed sites (Andrieu-Soler et al. 2005) further supports the fact that macrophages are essentially involved in the process leading to corneal lymphangiogenesis. However, it is known that IRS-1 is not only expressed by macrophages, and additional effects of Aganirsen on non-inflammatory cells might also contribute to the anti(lymph)angiogenic effect of this inhibitor. Indeed, we also observed a direct modulation of lymphatic endothelial cell proliferation by inhibition of IRS-1 signaling. Importantly, our study provides the first evidence that IRS-1 signaling modulates macrophage-derived (lymph)angiogenic growth factor expression and contributes to lymphatic vessel growth. The blockade of IRS-1 by Aganirsen is therefore a promising approach to treat corneal neovascular diseases, and Aganirsen has now been introduced in the clinical setting and is currently tested as eye drops in phase II and phase III trials. Initial results show that Aganirsen eye drops are able to inhibit progressive corneal neovascularization (Cursiefen et al. 2009). Furthermore, Aganirsen eye drops reduce the need for corneal transplantations in patients with herpetic keratitis-associated corneal neovascularization (Cursiefen et al. 2014). Aganirsen might be the first anti(lymph)angiogenic drug that will be approved for the topical treatment of corneal neovascularization.

3.3. An anti-inflammatory role of lymphatic vessels at the cornea?

It is generally accepted that corneal lymphatic vessels play a critical role in the induction and maintenance of various inflammatory diseases at the ocular surface, such as corneal graft rejection, dry eye disease, and, as we could recently

demonstrate, also in ocular allergy (Dietrich et al. 2010; Goyal et al. 2010; Lee et al. 2015). Therefore, corneal lymphangiogenesis is mostly considered as pathological, and the majority of corneal immunology and lymphvascular research is directed towards blocking lymphatic vessels (Bock et al. 2013; Hos et al. 2014). In contrast to this unfavorable role of corneal lymphatic vessels, a physiological, potentially *beneficial* function for corneal lymphatic vessels was not been described so far. Several studies in extraocular tissues had demonstrated that lymphatic vessels also exert important physiological functions during inflammatory reactions, because lymphatic vessels regulate tissue pressure and allow the drainage of debris and egress of inflammatory cells from the inflamed site (Oliver and Detmar 2002). In this context, lymphatic vessels have also been shown to contribute to the termination of ongoing inflammatory responses, and studies in the skin for instance indicate that the blockade of lymphangiogenesis might result in increased inflammatory edema formation and inflammatory cell accumulation, whereas the specific activation of lymphatic vessels might limit acute inflammation under certain circumstances (Huggenberger et al. 2011; Huggenberger et al. 2010). Here, we show for the first time that similar functions exist also for corneal lymphatic vessels, and that IL-10 is an important mediator of this putative anti-inflammatory lymphangiogenesis. Our results indicate that mainly anti-inflammatory polarized, IL-10 expressing macrophages display pro-lymphangiogenic properties. In addition, we have further demonstrated that inflammatory corneal lymphangiogenesis was reduced in IL-10 deficient mice and mice with conditional deletion of Stat3 in myeloid cells, which further supports our hypothesis that IL-10 regulates corneal lymphangiogenesis via macrophages. The pro-lymphangiogenic polarization of anti-inflammatory

macrophages, which seem to occur at later stages of the inflammatory response, could be an additional clearance mechanism to support the resolution of corneal inflammation: pro-lymphangiogenic macrophages might enhance the growth and draining capacity of lymphatic vessels, which in turn, support the egress of inflammatory cells and the termination of the local inflammatory response. Consistently, local treatment of inflamed corneas with IL-10 promoted the faster resolution of corneal inflammation. Taken together, our work provides evidence that IL-10 regulates corneal lymphangiogenesis, and that its effect is indirectly mediated through VEGF-C expressing corneal macrophages. Furthermore, our work supports the hypothesis of a novel beneficial and therapeutically applicable role of corneal lymphangiogenesis, which seems to be driven by anti-inflammatory macrophages.

3.4. Concluding remarks

During the past decade, anti(lymph)angiogenic treatment at the cornea has emerged as a reasonable and promising approach to treat a variety of inflammatory diseases of the ocular surface, e.g. corneal transplantation, dry eye disease, and ocular allergy (Bock et al. 2013). In these disease entities, lymphatic vessels seem to enable accelerated exit of APCs and antigen from the cornea, thereby facilitating the connection between the ocular surface and the regional lymph nodes, where accelerated allo- or autosensitization occurs (Hos and Cursiefen 2014; Stevenson et al. 2012). In this context, we and other groups investigated the impact of a “molecular lymphadenectomy”, namely a pharmacological blockade of lymphatic vessels, on disease outcome. Indeed, specific blockade of corneal lymphangiogenesis was sufficient to improve graft survival after corneal

transplantation or to ameliorate dry eye disease or ocular allergy, at least in the experimental setting (Dietrich et al. 2010; Goyal et al. 2012; Lee et al. 2015). Although glucocorticosteroid therapy is still widely used as the standard anti-inflammatory and also anti-(lymph)angiogenic treatment at the cornea, studies testing more specific anti-(lymph)angiogenic therapeutics have recently been translated into the clinic. In this regard, Bevacizumab is already widely used (off-label) in a variety of corneal neovascular diseases in patients and GS-101 (Aganirsen) eye drops have already entered phase III clinical trials (Bock et al. 2008; Cursiefen et al. 2014; Koenig et al. 2009).

The majority of corneal (lymph)vascular research indicates that corneal lymphangiogenesis is mostly undesirable. Thus, efforts are generally directed towards blocking lymphatic vessels. However, almost all studies showing a negative impact of corneal lymphangiogenesis on disease outcome dealt with ocular surface pathologies where the adaptive immune system is critically involved in disease induction or progression (such as corneal graft rejection, dry eye disease and ocular allergy). In these diseases, it is plausible that immunological processes in the secondary lymphoid organs contribute to disease outcome, and that inhibition of APC trafficking to the regional lymph nodes by anti-lymphangiogenic therapy is beneficial. However, we have shown here that corneal lymphangiogenesis might also have beneficial functions under certain circumstances, for instance in the termination of (sterile) inflammation. As the resolution of physiological inflammatory responses in the cornea seems to be fostered by the – possibly transient – presence of corneal lymphatic vessels, *activation* of corneal lymphangiogenesis, e.g. by pro-lymphangiogenic macrophages would open up a completely new

approach to promote corneal healing. Nevertheless, the specific mechanisms and mediators how macrophages contribute to corneal (lymph)angiogenesis and the functional consequences of this process are still not fully understood and require more detailed analysis, before macrophages can be used as specific targets to promote corneal repair and disease control.

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Curriculum Vitae

Personal data

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Scientific career

2002-2010	Medical School at the University of Erlangen-Nuremberg, Approbation (licence to practice medicine)
2006-2009	Study of Molecular Medicine at the University of Erlangen-Nuremberg, Diploma in Molecular Medicine
2006-2010	MD (summa cum laude), University of Erlangen-Nuremberg
2011-	Resident physician and scientific assistant, Department of Ophthalmology, University Hospital of Cologne
2012-	Enrollment into the MD/PhD program at the Center for Molecular Medicine Cologne (CMMC), University of Cologne
2013-	Management Committee Member, EU COST Action BM1302
2013-2015	GEROK research rotation, Medical Faculty, University of Cologne
2014-	Speaker, Arbeitsgruppe Young DOG, German Ophthalmological Society (DOG)
2014-	Principal Investigator, DFG FOR2240 (Lymph)angiogenesis and Cellular Immunity in Inflammatory Diseases of the Eye
2016	Fellow of the European Board of Ophthalmology (FEBO)

Prizes and honors

2007	Travel grant, GlaxoSmithKline GmbH, ARVO Annual Meeting 2007, Fort Lauderdale, USA
2007	Research scholarship of the Interdisciplinary Center of Clinical Research, IZKF, University of Erlangen-Nuremberg
2010	Karl-Giehrl-Award 2011 for best medical dissertation, University of Erlangen-Nuremberg
2013	Sicca-Research-Award, Annual Meeting of the German Ophthalmological Society (DOG) 2013, Berlin, Germany
2014	Research Award of the German Ophthalmological Society (Eye foundation)

Publications

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Erklärung

Ich habe Förderung erhalten.

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Hos D, Regenfuss B, Bock F, Onderka J, Cursiefen C. Blockade of insulin receptor substrate-1 inhibits corneal lymphangiogenesis. Invest Ophthalmol Vis Sci. 2011; 52(8):5778-85.

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Suppression of Inflammatory Corneal Lymphangiogenesis by Application of Topical Corticosteroids

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Objectives: To analyze whether topical application of corticosteroids inhibits inflammatory corneal lymphangiogenesis and to study the potential underlying antilymphangiogenic mechanisms.

Methods: Inflammatory corneal neovascularization was induced by suture placement, and the corneas were then treated with topical fluorometholone, prednisolone acetate, or dexamethasone sodium phosphate. After 1 week, the corneas were stained with lymphatic vessel endothelial hyaluronan receptor 1 for detection of pathological corneal lymphangiogenesis. The effect of these corticosteroids on macrophage recruitment was assessed via fluorescence-activated cell sorting analysis. The effect of these corticosteroids on proinflammatory cytokine expression by peritoneal exudate cells was tested via real-time polymerase chain reaction. Furthermore, the effect of steroid treatment on the proliferation of lymphatic endothelial cells was assessed via enzyme-linked immunosorbent assay.

Results: Treatment with corticosteroids resulted in a significant reduction of inflammatory corneal lymphangiogenesis. The antilymphangiogenic effect of fluorometholone was significantly weaker than that of prednisolone and dexamethasone. Corneal macrophage recruitment was also significantly inhibited by the application of topical steroids. Treatment of peritoneal exudate cells with corticosteroids led to a significant downregulation of the RNA expression levels of tumor necrosis factor and interleukin 1β. Additionally, proliferation of lymphatic endothelial cells was also inhibited.

Conclusions: Corticosteroids are strong inhibitors of inflammatory corneal lymphangiogenesis, with significant differences between various corticosteroids in terms of their antilymphangiogenic potency. The main mechanism of the antilymphangiogenic effect seems to be through the suppression of macrophage infiltration, proinflammatory cytokine expression, and direct inhibition of proliferation of lymphatic endothelial cells.

Clinical Relevance: Steroids block corneal lymphangiogenesis, the main risk factor for immune rejections after corneal transplantation. The different antilymphangiogenic potency of these drugs should be taken into account when using steroids in clinical practice (eg, after keratoplasty).

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THE HEALTHY CORNEA IS DEVOID of both blood and lymphatic vessels and actively maintains its avascularity.[1-3] However, a variety of inflammatory conditions can lead to a breakdown of this "angiogenic privilege." This leads to the outgrowth of blood as well as lymphatic vessels from the limbus into the avascular cornea, reducing transparency and visual acuity.[4-6] Furthermore, corneal neovascularization is also the most important risk factor for graft rejection after corneal transplantation; in particular, lymphangiogenesis has been shown to be essential in mediating immune reactions after corneal grafting.[4,7-9] By use of the murine model of corneal transplantation, it has recently been shown that inhibition of corneal neovascularization after low- and high-risk corneal transplantation promotes graft survival.[10-13] Thus, antiangiogenic therapy is a very reasonable approach for reducing corneal complications secondary to inflammation and also for preventing immune rejections after corneal transplantation.

Recently, several specific angiogenesis inhibitors have been approved by the US Food and Drug Administration for the treatment of pathologic neovascularization in the eye. Ranibizumab (Lucentis; Genentech, South San Francisco, California) and pegaptanib (Macugen; OSI Pharmaceuticals, Farmingdale, New York) were both approved for the treatment of age-related macular degeneration. Bevacizumab (Avastin; Genentech, South San Francisco, California), approved for co-

lorectal cancer and nonsmall cell lung cancer treatment, is also widely used off-label to treat age-related maculopathy, proliferative retinopathies, and neovascular glaucoma.[14] In contrast, no specific angiogenesis inhibitor against neovascularization at the anterior segment of the eye has been approved by the US Food and Drug Administration so far.[15]

However, many older, established drugs also have antiangiogenic properties in addition to their known activities. For instance thalidomide, spironolactone, nonsteroidal anti-inflammatory drugs, and corticosteroids are well-known drugs with known toxicity profiles, and it has been shown that these substances can inhibit angiogenesis in various immune and inflammatory diseases, including those of the eye.[16-21] Corticosteroids are potent anti-inflammatory drugs widely used for the treatment of macular edema and choroidal and retinal neovascularization.[22] To date, corticosteroid therapy is also the standard anti-inflammatory and antiangiogenic treatment for patients with corneal neovascularization, especially after corneal transplantation.[15,23,24]

Although it has been established that corticosteroids are able to inhibit corneal hemangiogenesis,[15,21,23,25] little is known about their ability to suppress corneal lymphangiogenesis.[26,27] Nakao and colleagues[21] showed that systemic application of dexamethasone sodium phosphate was able to inhibit interleukin 1β (IL-1β)–induced corneal hemangiogenesis. However, to our knowledge, it has not been shown whether corticosteroids are also able to inhibit corneal lymphangiogenesis. Therefore, the aims of our study were to assess whether topical application of corticosteroids is able to inhibit inflammatory corneal lymphangiogenesis and to compare the antilymphangiogenic potential of various clinically used corticosteroids (fluorometholone, prednisolone acetate, and dexamethasone). Furthermore, we analyzed the effect of steroids on inflammatory cell recruitment and proinflammatory cytokine expression by macrophages, which are known to be crucial mediators of inflammatory corneal lymphangiogenesis.[28] In addition, we investigated whether steroids are also able to directly suppress the proliferation of lymphatic endothelial cells (LECs).

METHODS

ANIMALS AND ANESTHESIA

All animal protocols were approved by the local animal care committee and were in accordance to the Association for Research in Vision and Ophthalmology's Statement for the Use of Animals in Ophthalmology and Vision Research. Mice were anesthetized with an intraperitoneal injection of a combination of 8 mg/kg of ketamine hydrochloride and 0.1 ml/kg of xylazine hydrochloride. All mice were female BALB/c mice, aged 6 to 8 weeks (purchased from Charles River Laboratories, Sulzfeld, Germany).

SUTURE-INDUCED, INFLAMMATORY CORNEAL NEOVASCULARIZATION ASSAY

The mouse model of suture-induced inflammatory corneal neovascularization was used as previously described.[13,28,29] Prior to corneal neovascularization, each animal was deeply anesthetized. The cornea actively maintains its avascularity; in particular, the corneal epithelium contains antiangiogenic molecules such as soluble vascular endothelial growth factor receptor 1 and membrane bound vascular endothelial growth factor receptor 3.[2,3] Therefore, to obtain higher angiogenic and inflammatory response rates, the central cornea was marked with a 2.0-mm diameter trephine and de-epithelialized before suture placement. Three 11-0 nylon sutures (Serag Wiessner, Naila, Germany) were then placed intrastromally with 2 stromal incursions extending over 120° of corneal circumference each. The outer point of suture placement was chosen near the limbus, and the inner suture point was chosen near the corneal center equidistant from limbus to obtain standardized angiogenic responses. Sutures were left in place for 7 days. Treatment groups were as follows: fluorometholone, prednisolone, and dexamethasone (each at a dosage of 1 mg/mL, 3 eye drops daily, 5 µL per drop). It has previously been shown that when fluorometholone, prednisolone, and dexamethasone are all formulated at a concentration of 0.1%, the intraocular penetration of these steroids is almost identical.[30] Therefore, we decided to use all steroids at a concentration of 0.1%, although prednisolone is clinically used at a concentration of 1%. Control mice received equal amounts of saline solution. After 1 week, mice were killed and corneas were prepared. The corneal neovascularization assay included 12 mice per group.

CORNEAL WHOLE MOUNTS AND MORPHOLOGICAL DETERMINATION OF LYMPHANGIOGENESIS AND HEMANGIOGENESIS

The excised corneas from the corneal neovascularization assay were rinsed in phosphate-buffered saline (PBS) and fixed in acetone for 30 minutes. After 3 washing steps in PBS and blocking with 2% bovine serum albumin in PBS for 2 hours, the corneas were stained overnight at 4°C with rabbit anti–mouse lymphatic vessel endothelial hyaluronan receptor 1 (1:500; AngioBio Co, Del Mar, California) and rat anti–mouse CD31-FITC (1:50; Acris Antibodies GmbH, Hiddenhausen, Germany). On day 2, the tissue was washed 3 times; lymphatic vessel endothelial hyaluronan receptor 1 was then detected with a Cy3-conjugated secondary goat anti–rabbit antibody (1:100; Dianova, Hamburg, Germany). After 3 additional washing steps in PBS, all corneas were moved to Superfrost slides (Menzel-Gläser, Braunschweig, Germany) and covered with Dako fluorescent mounting medium (Hamburg, Germany) and stored at 4°C in the dark.

Stained whole mounts were analyzed with a fluorescence microscope (BX51; Olympus Optical Co, Hamburg, Germany), and digital pictures were taken with a 12-bit monochrome charge-coupled device camera (F-View II; Soft Imaging System, Münster, Germany). Each whole-mount picture was assembled out of 9 pictures taken at 100× magnification. The areas covered with lymphatic and blood vessels were detected with an algorithm established in the image-analyzing program cell^F (Soft Imaging System, Münster, Germany): prior to analysis, gray-value images of the whole-mount pictures were modified by several filters, and vessels were detected by threshold setting, including the bright vessels and excluding the dark background. A detailed explanation of this method was described previously.[31] The mean vascularized area of the control whole mounts was defined as being 100%, and the vascularized areas were then related to this value.

FLOW CYTOMETRIC ANALYSIS OF INFLAMMATORY CORNEAL CELL RECRUITMENT

Corneal inflammation was induced by suture placement, and corneas were then treated with topical fluorometholone, pred-

nisolone, dexamethasone (each at a dosage of 1 mg/mL, 3 eye drops daily, 5 µL per drop), or saline solution (control). On days 2 and 5 after suture placement, corneas (3 per group) were harvested and pooled. Single-cell suspensions were prepared from corneal samples using collagenase digestion, as previously described.[32] Briefly, corneal buttons were removed and minced into small fragments, followed by digestion with 2 mg/mL of type IV collagenase (Sigma-Aldrich, St Louis, Missouri) and 0.05 mg/mL of deoxyribonuclease I (Roche, Basel, Switzerland) for 1 hour at 37°C with agitation. The suspension was then triturated through a 10-mL syringe to homogenize the remaining tissue and filtered through a 70-µm cell strainer. Once in single-cell suspension, all samples underwent Fc receptor blockade via incubation with α-CD16/CD32 (BD Pharmingen, San Diego, California) at 4°C in 0.5% bovine serum albumin (Sigma-Aldrich). Subsequent antibody labeling included α-CD11b (BD Pharmingen) and α-F4/80 (BD Pharmingen). All antibodies were analyzed with the appropriate isotype controls.

COLLECTION, CULTURE, AND TREATMENT OF PERITONEAL MACROPHAGES

Thioglycollate-induced macrophages were collected from the peritoneal cavity (peritoneal exudate cells) of 8-week-old female mice as described previously.[33] Peritoneal exudate cells were washed, resuspended, and cultured at 37°C in RPMI-1640 medium containing 10% fetal calf serum, 10mM HEPES, 1mM nonessential amino acids, 1mM sodium pyruvate, 2mM L-glutamine, 100-U/mL penicillin, and 100-mg/mL streptomycin. After adhesion, nonadherent cells were removed by washing with culture medium, and adherent cells were then used as macrophages. Cells collected by this method are F4/80+ (>90%) and CD11b+ (>99%).[33,34]

Cells were incubated in RPMI-1640 medium containing 25 nmol/L of fluorometholone, prednisolone, or dexamethasone for 24 hours, followed by RNA isolation. To analyze RNA expression under higher inflammatory conditions, 50 ng/mL of tumor necrosis factor (TNF; Biomol GmbH, Hamburg, Germany) was added in some experiments, and cells were then incubated for 24 hours with or without 25 nmol/L of dexamethasone.

RNA ISOLATION AND REAL-TIME POLYMERASE CHAIN REACTION

RNA from cultured peritoneal macrophages was isolated with the RNeasy Micro kit (Qiagen, Hilden, Germany). Complementary DNA was synthesized with random hexamers using reverse transcriptase (SuperScript III; Invitrogen, Darmstadt, Germany) according to the manufacturer's recommendations. Real-time polymerase chain reaction (PCR) was performed using TaqMan Universal PCR Mastermix and preformulated primers for TNF (assay Mm99999068_m1), IL-1β (assay Mm00434228_m1), and *GAPDH* (assay Mm99999915_g1); FAM-MGB dye-labeled predesigned primers were used for TNF, IL-1β, and *GAPDH* (Applied Biosystem, Foster City, California). The results were analyzed by the comparative threshold cycle method and normalized by *GAPDH* as an internal control.

The relative messenger RNA (mRNA) level in the untreated group was used as the normalized control for the treatment groups. All assays were performed in duplicate; a nontemplate control was included in all of the experiments to evaluate DNA contamination of the reagents used. Experiments were conducted twice.

LEC PROLIFERATION ENZYME-LINKED IMMUNOSORBENT ASSAY

The LEC proliferation enzyme-linked immunosorbent assay (ELISA) was used, with slight modifications, as previously described.[13] Human lymphatic microvascular endothelial cells (Cambrex Bio Science, Walkersville, Maryland) were cultured in EGM2-MV full medium (Cambrex Bio Science) according to the manufacturer's instructions; EGM2-MV full medium contains endothelial cell growth factors such as vascular endothelial growth factor and basic fibroblast growth factor. For this ELISA, cells were seeded in a 96-well plate in EGM2-MV medium at a density of 4×10^3 cells per well. After 6 hours, the medium was replaced with a serum-free medium, 10 µL/mL of 5-bromodeoxyuridine (BrdU; Cell Proliferation ELISA, BrdU, Roche, Penzberg, Germany), and the corticosteroids (25 nmol/L of fluorometholone, prednisolone, or dexamethasone) were added. Cells were fixed and stained after 48 hours according to the manufacturer's instructions. Colorimetric analysis was performed with the ELISA reader SLT Spectra (SLT Labinstruments Deutschland GmbH, Crailsheim, Germany). The mean extinction of the control wells was defined as being 100%, and extinction of all wells were then related to this value (LEC proliferation ratio).

STATISTICAL ANALYSIS

Statistical analyses were performed using by Microsoft Excel 2000 (Microsoft Corp, Redmond, Washington) and InStat version 3.06 (GraphPad Software Inc, San Diego, California). Statistical significance was determined using the 1-way analysis of variance test. $P < .05$ was considered statistically significant. Graphs were drawn using Prism4 version 4.03 (GraphPad Software Inc).

RESULTS

INHIBITORY EFFECT OF TOPICAL TREATMENT WITH CORTICOSTEROIDS ON CORNEAL LYMPHANGIOGENESIS AND HEMANGIOGENESIS IN VIVO

Treatment with corticosteroids resulted in a significant reduction of inflammatory corneal lymphangiogenesis in vivo. In comparison with controls, lymphangiogenesis was inhibited by 33% via topical fluorometholone treatment (mean [SD], 67% [19%]; $P < .001$), by 53% via topical prednisolone treatment (mean [SD], 47% [17%]; $P < .001$), and by 55% via topical dexamethasone treatment (mean [SD], 45% [13%]; $P < .001$). The inhibitory effect of fluorometholone was significantly weaker than the inhibitory effect of prednisolone or dexamethasone ($P < .01$). Every tested steroid showed very similar effects on corneal hemangiogenesis: In comparison with controls, blood vessel growth was inhibited by 30% via fluorometholone treatment (mean [SD], 70% [9%]; $P < .001$), by 50% via topical prednisolone treatment (mean [SD], 50% [12%]; $P < .001$), and by 57% via topical dexamethasone treatment (mean [SD], 43% [7%]; $P < .001$). The inhibitory effect of fluorometholone was again significantly weaker than the inhibitory effect of prednisolone or dexamethasone ($P < .01$) (**Figure 1**).

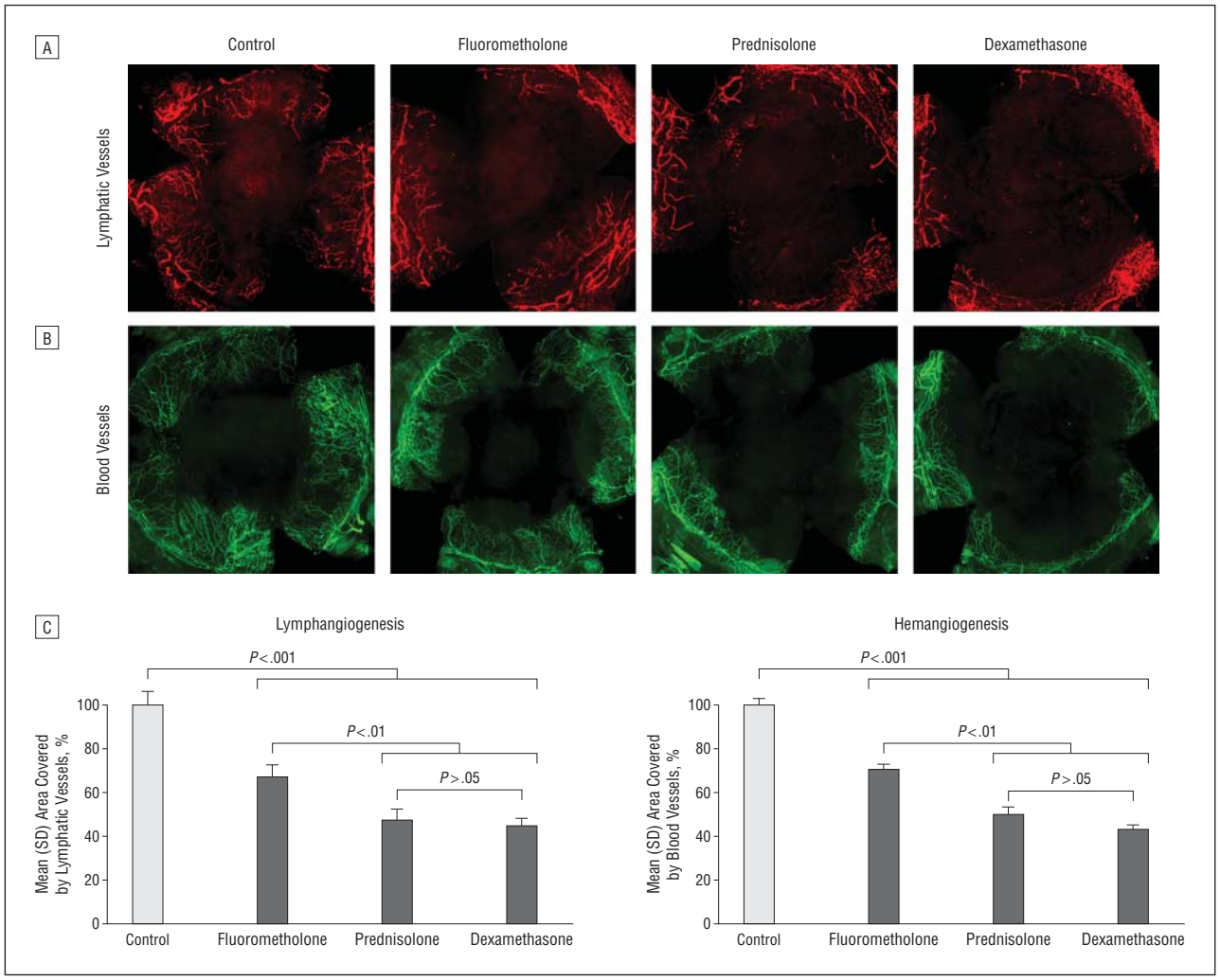

Figure 1. Suppressive effect of corticosteroids on inflammatory corneal lymphangiogenesis in vivo. A, Corneal whole mounts, stained with lymphatic vessel endothelial hyaluronan receptor 1; B, corneal whole mounts, stained with CD31; and C, quantification of lymphangiogenesis and hemangiogenesis. Both lymphangiogenesis and hemangiogenesis were significantly inhibited by topical application of fluorometholone, prednisolone acetate, or dexamethasone sodium phosphate.

INHIBITORY EFFECT OF TOPICAL TREATMENT WITH CORTICOSTEROIDS ON INFLAMMATORY CELL RECRUITMENT IN VIVO

To examine the effect of topical corticosteroid treatment on suture-induced macrophage infiltration, we quantified the number of F4/80+CD11b+ cells using flow cytometry. Fluorescence-activated cell sorting analysis showed that topical treatment with corticosteroids resulted in a significant reduction of inflammatory cell recruitment in vivo. Although, in control corneas, the percentage of F4/80+CD11b+ macrophages per cornea was 23.58% at 2 days after corneal suture placement and 27.14% at 5 days after corneal suture placement, treatment with fluorometholone reduced the amount of F4/80+CD11b+ cells to 2.07% at day 2 and 13.57% at day 5. Treatment with prednisolone reduced the amount of F4/80+CD11b+ cells to 4.51% at day 2 and 10.44% at day 5, and treatment with dexamethasone reduced the amount of F4/80+CD11b+ cells to 1.88% at day 2 and 11.86% at day 5. All of the steroids used led to a stronger inhibitory effect on macrophage recruitment on day 2 than on day 5 (**Figure 2**).

SUPPRESSIVE EFFECT OF CORTICOSTEROIDS ON PROINFLAMMATORY CYTOKINE EXPRESSION BY MACROPHAGES IN VITRO

Peritoneal exudate cells were incubated with 25-nmol/L fluorometholone, prednisolone, or dexamethasone for 24 hours, and mRNA expression levels of the proinflammatory cytokines TNF and IL-1β were then measured via real-time PCR. In vitro treatment of resting peritoneal exudate cells with corticosteroids led to a significant inhibition of mRNA expression levels of TNF and IL-1β. Fluorometholone treatment inhibited mRNA expression levels by 83% (TNF) and 68% (IL-1β), whereas prednisolone treatment suppressed mRNA expression levels by 79% (TNF) and 50% (IL-1β). Dexamethasone was the strongest inhibitor of proinflammatory cytokine expression: TNF expression was reduced by 88%, and IL-1β expression by 73%. With respect to TNF expression levels, there were no significant differences detectable between fluorometholone treatment and prednisolone treatment ($P>.05$) or between fluorometholone treatment and dexamethasone treatment ($P>.05$). However, treatment with

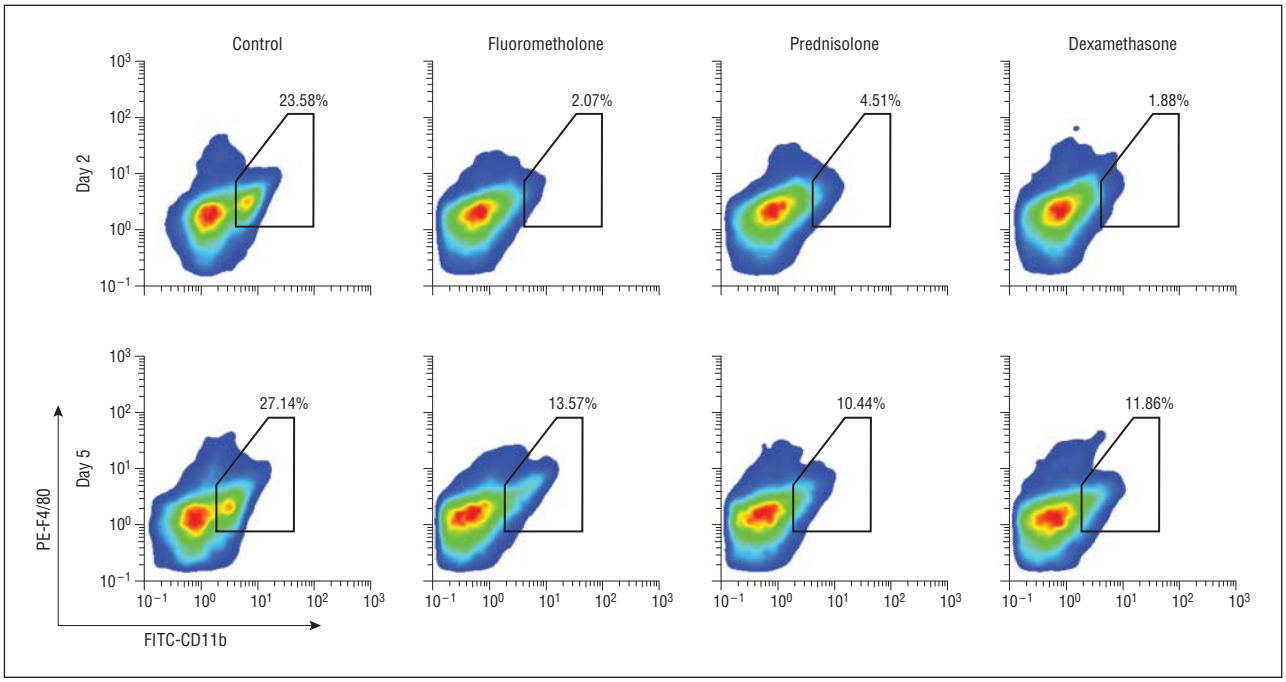

Figure 2. Inhibitory effect of topical treatment with corticosteroids on inflammatory cell recruitment in vivo. Fluorescence-activated cell sorting analysis of F4/80+CD11b+ cells obtained from corneas after suture placement. Cell recruitment was strongly inhibited by topical fluorometholone, prednisolone acetate, or dexamethasone sodium phosphate. All of the steroids used led to a stronger inhibition of cell recruitment on day 2 than on day 5. FITC indicates fluorescein isothiocyanate; and PE, phycoerythrin.

dexamethasone showed a statistically stronger inhibition of TNF mRNA expression than did treatment with prednisolone ($P<.05$). The assessment of IL-1β mRNA levels revealed that both fluorometholone and dexamethasone had a stronger inhibitory effect on IL-1β expression than did prednisolone ($P<.01$), with the effect of fluorometholone and dexamethasone being comparable ($P>.05$).

To analyze whether steroids can also inhibit TNF and IL-1β expression under higher inflammatory conditions, peritoneal exudate cells were incubated with 50 ng/mL of TNF. The addition of this inflammatory stimulus led to a strong upregulation of proinflammatory cytokine expression (a 4.6-fold increase in TNF expression and a 4.4-fold increase in IL-1β expression). However, further treatment with dexamethasone could still suppress proinflammatory cytokine expression, even under this inflammatory stimulation (a 0.3-fold decrease TNF expression and a 0.4-fold decrease IL-1β expression) (**Figure 3**).

SUPPRESSIVE EFFECT OF CORTICOSTEROIDS ON LEC PROLIFERATION IN VITRO

To assess whether corticosteroids have a direct effect on LECs as well, we studied their effect on LEC proliferation in vitro. Treatment with corticosteroids (dose, 25 nmol/L) significantly suppressed the proliferation of LECs. Compared with controls, application of fluorometholone resulted in an inhibition of LEC proliferation by 42% ($P<.001$), application of prednisolone resulted in an inhibition of LEC proliferation by 30% ($P<.001$), and application of dexamethasone resulted in an inhibition of LEC proliferation by 51% ($P<.001$). Between the analyzed steroids, prednisolone had the weakest effect on LEC proliferation (prednisolone vs fluorometholone [$P<.01$]; prednisolone vs dexamethasone [$P<.001$]). Dexamethasone was the strongest inhibitor of LEC proliferation (dexamethasone vs fluorometholone; $P<.05$) (**Figure 4**).

COMMENT

The experiments performed in our study demonstrate the following: (1) Corticosteroids are potent inhibitors of inflammation-induced lymphangiogenesis and hemangiogenesis in vivo. (2) Significant differences exist between different corticosteroids with respect to their antilymphangiogenic and antihemangiogenic properties. In general, the stronger the anti-inflammatory effect, the stronger the in vivo antilymphangiogenic and antihemangiogenic effect. This fits well with the known close interrelation between inflammation and angiogenesis. (3) Corticosteroids strongly block inflammatory cell recruitment into the inflamed cornea. It has already been shown that systemic application of dexamethasone was able to inhibit the recruitment of CD11b single positive cells after IL-1β pellet implantation.[21] We analyzed the effect of topical steroid treatment on F4/80+CD11b+ double-positive cells after corneal suture placement in order to primarily focus on the role of macrophages, because various leukocyte populations (eg, granulocytes and natural killer cells) also express CD11b, whereas F4/80 seems to be mainly expressed by macrophages.[35,36] Furthermore, compared with pellet implantation, the suture-induced neovascularization model used in our study is known to induce a strong inflammatory response. The inhibition of F4/80+CD11b+ macrophage infiltration by steroids af-

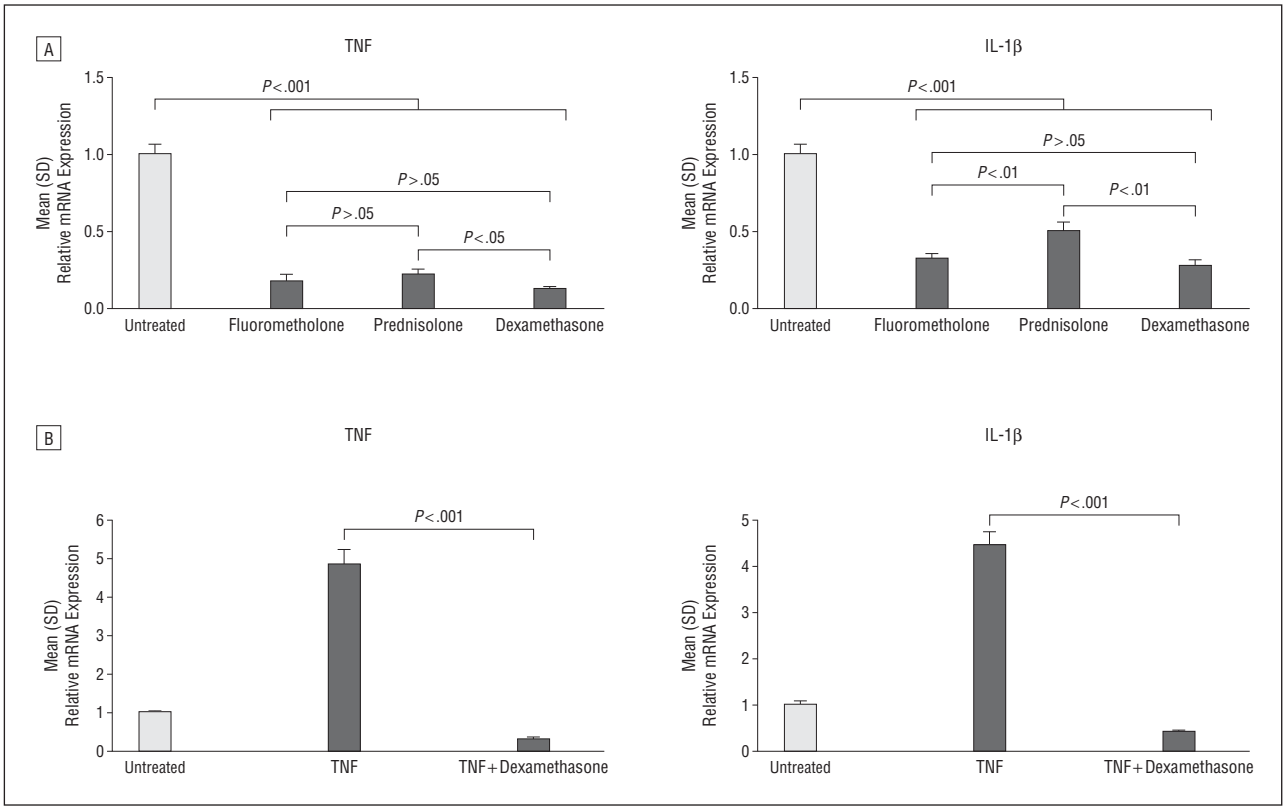

Figure 3. Suppressive effect of corticosteroids on proinflammatory cytokine expression by macrophages in vitro. Treatment of peritoneal exudate cells with corticosteroids led to a significant inhibition of mRNA expression levels of tumor necrosis factor (TNF) and interleukin 1β (IL-1β), both under resting (A) and inflammatory conditions (B).

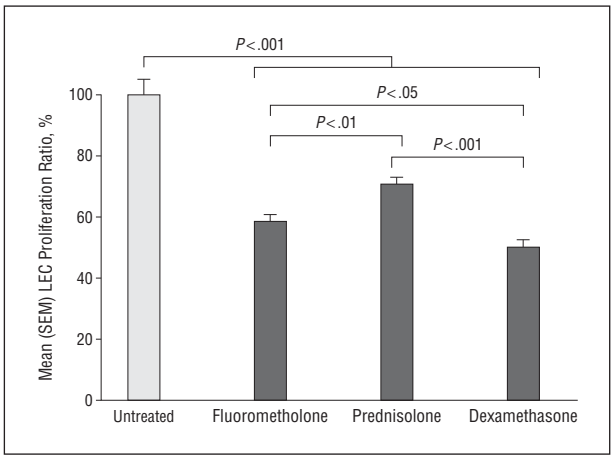

Figure 4. Inhibitory effect of corticosteroids on proliferation of lymphatic endothelial cells (LECs) in vitro. Proliferation was measured by a cell proliferation enzyme-linked immunosorbent assay with 5-bromodeoxyuridine.

ter suture placement allowed us to conclude that, even under these very high inflammatory conditions, corticosteroids strongly block macrophage recruitment after topical application. (4) In vitro, corticosteroids significantly inhibit proinflammatory cytokine expression by macrophages. It is well established that both TNF and IL-1β mediate corneal neovascularization.[37,38] In particular, TNF expressed by macrophages is known to be an important factor that induces angiogenesis.[39] We could show that steroids are able to suppress proinflammatory cytokine expression, both under resting and inflammatory conditions. Therefore, blockade of macrophage-derived expression of TNF and IL-1β levels by steroids likely contributes to the strong antilymphangiogenic effect of these substances. (5) Moreover, corticosteroids also suppress LEC proliferation, indicating that the antilymphangiogenic effect of these substances is due not only to anti-inflammatory properties but also partly to direct anti-proliferative properties.

Fluorometholone, the weakest of the 3 anti-inflammatory corticosteroids tested, had the least effect on corneal lymphangiogenesis in vivo; however, it did have a stronger inhibitory effect than did prednisolone in vitro. Prednisolone had significantly stronger inhibitory effects in vivo; however, it had the weakest inhibitory effect in vitro. Therefore, additional properties (eg, tissue and cell penetration and substance half-life) also seem to be responsible for the overall inhibitory effect in vivo. Indeed, it has been shown that fluorometholone has a relatively short half-life and rapid metabolism.[40] Dexamethasone was the most potent inhibitor in almost all conducted experiments. The different antilymphangiogenic potentials of these 3 corticosteroids should be taken into account when using them in clinical practice (eg, after keratoplasty). Also, however, the risk of adverse effects seems to decrease in parallel with the anti-inflammatory potency of the particular steroid. For example, it has been established that fluorometholone increases intraocular pressure less frequently than does prednisolone or dexamethasone. The benefit of a steroid with higher anti-inflammatory and, as shown in our study, also higher

antiangiogenic properties surely has to be weighed against its increasing risk of adverse effects in patients.

A plethora of clinical indications exist for antiangiogenic treatment of the cornea (eg, to stop sight-threatening neovascularization after inflammation or to improve graft survival after corneal transplantation).[4,41-45] To date, steroid therapy is the standard anti-inflammatory and antiangiogenic treatment for patients with corneal neovascularization, especially after corneal transplantation.[15] Furthermore, it is widely accepted that topical steroid treatment protects against immunologic graft rejections after corneal transplantation.[23,24] This protection may largely be attributable to the fact that corticosteroids are very potent anti-inflammatory substances. The fact that these substances are also able to inhibit lymphangiogenesis, as shown in our study, and that lymphangiogenesis has been shown to be an important risk factor regarding graft rejection after corneal transplantation[4,7] may lead one to conclude that both the anti-inflammatory effects and the antilymphangiogenic properties of these substances can result in a better outcome for patients after corneal transplantation.

However, the use of steroid therapy in the management of eye diseases remains controversial because of the adverse effects associated with this type of therapy.[23,46] Alternative therapeutic approaches are necessary; for example, specifically blocking angiogenesis by targeting vascular endothelial growth factor appears to be a very reasonable and promising approach with less adverse effects.[14,47] In fact, initial successful results were obtained using specific antiangiogenic drugs at the cornea (eg, bevacizumab eye drops and GS-101 antisense oligonucleotide eye drops).[48,49]

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Blockade of Insulin Receptor Substrate-1 Inhibits Corneal Lymphangiogenesis

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Purpose. To analyze whether insulin receptor substrate (IRS-1) is involved in lymphatic vessel development and whether IRS-1 blockade can inhibit lymphangiogenesis in vivo.

Methods. The impact of IRS-1 blockade by GS-101 (Aganirsen), an antisense oligonucleotide against IRS-1, on lymphatic endothelial cell (LEC) proliferation was assessed by ELISA. Furthermore, the effect of IRS-1 blockade on prolymphangiogenic growth factor expression by LECs and macrophages (peritoneal exudate cells) was tested by real-time PCR. The mouse model of inflammatory corneal neovascularization was used to analyze the effect of IRS-1 blockade in vivo: after corneal suture placement, mice were treated with GS-101 eye drops (twice daily afterwards for 1 week, 5 μL per drop; 50, 100, or 200 μM). Afterward, corneal wholemounts were prepared and stained for blood and lymphatic vessels.

Results. Blockade of IRS-1 by GS-101 inhibited LEC proliferation dose dependently. GS-101 led to decreased VEGF-A expression levels in LECs, whereas VEGF-C, VEGF-D, and VEGFR3 showed no significant change. In macrophages, VEGF-A expression levels were also inhibited by IRS-1 blockade. Additionally, GS-101 strongly inhibited macrophage-derived VEGF-C, VEGF-D, and VEGFR3 expression. In vivo, corneal hemangiogenesis was significantly inhibited when used at a concentration of 200 μM (by 17%; $P < 0.01$). Corneal lymphangiogenesis was significantly inhibited when used at a dose of 100 μM (by 21%; $P < 0.01$), and the highest used dose (200 μM) showed an even stronger inhibition (by 28%; $P < 0.001$).

Conclusions. Blockade of IRS-1 inhibits not only hemangiogenesis but also lymphangiogenesis. To the authors' knowledge, this is the first evidence that IRS-1 is involved in the molecular pathway leading to lymphangiogenesis. (*Invest Ophthalmol Vis Sci.* 2011;52:5778–5785) DOI:10.1167/iovs.10-6816

Lymphangiogenesis, the development and growth of lymphatic vessels, occurs under a variety of pathologic conditions, such as chronic inflammation and graft rejection, and plays a crucial role in cancer growth and dissemination.[1–3] This process is driven by the production of prolymphangiogenic growth factors and proinflammatory cytokines such as VEGF-A, VEGF-C, VEGF-D, FGF, TNF-α, and IL-1β.[4–9] Subsequent binding of these factors to their receptors initiates complex signaling cascades, and the involvement of numerous molecules and many different cell types makes it challenging to fully understand the process of lymphatic vessel development but also offers many therapeutic targets to interfere with and inhibit lymphangiogenesis.

Insulin receptor substrate (IRS-1) is a cytosolic adapter protein without intrinsic kinase activity.[10] The main function of this protein is to recruit other proteins to their receptors and induce the organization of intracellular signaling cascades. IRS-1 was originally isolated as an insulin receptor substrate but has since been shown to work as a proximal scaffold protein in a broad variety of growth hormone and cytokine receptor signaling cascades.[10] The role of IRS-1 in angiogenesis seems to be through its interaction with the VEGF-receptor complex.[11] Furthermore, it has been shown that IRS-1 is able to interact with integrins, multifunctional proteins also involved in lymphangiogenesis.[12–14]

Several studies have investigated the role of IRS-1 signaling in hemangiogenesis.[15–17] IRS-1 expression in endothelial cells is upregulated under angiogenic conditions.[17] Furthermore, hypoxic retinal neovascularization is reduced in IRS-1 knockout mice.[15] Moreover, IRS-1 is also expressed in the cornea, and it has recently been shown that GS-101 (Aganirsen), an antisense oligonucleotide that blocks the expression of IRS-1, inhibits corneal hemangiogenesis, both in experimental and clinical settings.[16,18] Studies addressing the underlying antiangiogenic mechanisms showed that GS-101 inhibited endothelial tube-like structure formation and VEGF-A and IL-1β expression by endothelial cells.[17]

However, it is thus far unclear whether IRS-1 signaling is involved in the molecular pathway leading to *lymphatic* vessels. Therefore, the aim of this study was to investigate whether the blockade of IRS-1 signaling by GS-101 is also able to suppress *lymph*angiogenesis. We analyzed the impact of IRS-1 blockade on lymphatic endothelial cell (LEC) proliferation and prolymphangiogenic factor expression by LECs and macrophages, which have been shown to be essential mediators of lymphangiogenesis.[19,20] Finally, we analyzed the effect of GS-101 eye drops on inflammatory corneal *lymph*angiogenesis in vivo.

Methods

Lymphatic Endothelial Cell Culture, Treatment, and Proliferation ELISA

LEC proliferation ELISA was used, with slight modifications, as previously described.[21] Briefly, human lymphatic microvascular endothelial cells (Cambrex Bio Science, Walkersville, MD) were cultured in EGM2-MV full medium (EGM 2-MV full medium contains endothelial cell growth factors such as VEGF and bFGF). For ELISA, cells were seeded in a 96-well plate in EGM2-MV medium at a density of 4×10^3 cells/well. Six hours after seeding, medium was replaced with EGM2-MV minimal medium (without growth factors), BrdU (10 μL/mL;

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Cell Proliferation ELISA, BrdU, Roche, Indianapolis, IN), and GS-101 at various concentrations were then added. Cells were fixed and stained after 48 hours according to manufacturer's instructions. Colorimetric analysis was performed with an ELISA reader (Multiskan Spectrum; Thermo Electron Corporation, Waltham, MA). The mean extinction of the control wells was defined as 100%; the extinction of all wells was then related to this value (LEC proliferation ratio). For RNA expression analyses, LECs were incubated in EGM2-MV full medium containing various concentrations of GS-101 for 24 hours, followed by RNA extraction.

Collection, Culture, and Treatment of Peritoneal Macrophages

Thioglycollate-induced peritoneal exudate cells (PECs) were collected from the peritoneal cavities of 8- to 10-week-old female mice, as described previously.[22] PECs were washed, resuspended, and cultured at 37°C in RPMI 1640 medium containing 10% FCS, 10 mM HEPES, 1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin. After adhesion, nonadherent cells were removed by washing with culture medium, and adherent cells were then used as macrophages. Cells collected by this method are F4/80$^+$ (>90%) and CD11b$^+$ (>99%).[20,22] For RNA expression analyses, cells were incubated in RPMI 1640 medium containing various concentrations of TNF-α, IL-1β (both 10 ng/mL), or GS-101 for 24 hours, followed by RNA isolation.

RNA Isolation and Real-Time PCR

RNA from LECs and PECs was isolated with an RNA purification kit (RNeasy Micro Kit; Qiagen, Valencia, CA). Complementary DNA (cDNA) was synthesized with random hexamers using reverse transcriptase (SuperScript III; Invitrogen, Carlsbad, CA). Primer (MWG Biotech, Ebersberg, Germany) was designed using Primer3 software and BLAST (Basic Local Alignment Search Tool, National Center for Biotechnology Information). PCR reactions (20 μL) contained 10 to 20 ng cDNA (depending on the analyzed gene), 0.4 μM of each forward and reverse primer, and master mix (SsoFast EvaGreen Supermix; Bio-Rad, Hercules, CA). Real-time PCR was performed under the following conditions: initial denaturation step of 95°C for 2 minutes, 40 cycles of 95°C for 5 seconds and of 56°C to 62.4°C (depending on the analyzed gene) for 15 seconds, followed by an additional denaturation

TABLE 1. Primers Used for Real-Time PCR

Gene	Product Size (bp)	Annealing Temperature (°C)	Sequence (5′–3′)
HPRT1 (human)	162	56	F: CCTGGCGTCGTGATTAGTG R: GCCTCCCATCTCCTTCATC
VEGF-A (human)	167	56	F: ACAGGTACAGGGATGAGGACAC R: AAGCAGGTGAGAGTAAGCGAAG
VEGF-C (human)	163	56	F: GCCTGTGAATGTACAGAAAGTCC R: AATATGAAGGGACACAACGACAC
VEGF-D (human)	97	56	F: CCGCCATCCATACTCAATTATC R: CCATAGCATGTCAATAGGACAGAG
VEGFR3 (human)	109	56	F: GGTACATGCCAACGACACAG R: CTCAAAGTCTCTCACGAACACG
HPRT (mouse)	163	58.5–62.4	F: GTTGGATACAGGCCAGACTTTGTTG R: GATTCAACTTGCGCTCATCTTAGGC
IRS-1 (mouse)	122	58.5	F: GACGCTCCAGTGAGGATTTAAG R: GGATTTGCTGAGGTCATTTAGG
VEGF-A (mouse)	184	60	F: CATGGATGTCTACCAGCGAAG R: CATGGTGATGTTGCTCTCTGAC
VEGF-C (mouse)	219	60	F: AGAACGTGTCCAAGAAATCAGC R: ATGTGGCCTTTTCCAATACG
VEGF-D (mouse)	86	62.4	F: ATGGCGGCTAGGTGATTCC R: CCCTTCCTTTCTGAGTGCTG
VEGFR3 (mouse)	94	60	F: GTCCCTCTACTTCCAACTGCTTC R: CACTCCTCCTCTGTGACTTTGAG
HPRT1 (human)	162	56	F: CCTGGCGTCGTGATTAGTG R: GCCTCCCATCTCCTTCATC
VEGF-A (human)	167	56	F: ACAGGTACAGGGATGAGGACAC R: AAGCAGGTGAGAGTAAGCGAAG
VEGF-C (human)	163	56	F: GCCTGTGAATGTACAGAAAGTCC R: AATATGAAGGGACACAACGACAC
VEGF-D (human)	97	56	F: CCGCCATCCATACTCAATTATC R: CCATAGCATGTCAATAGGACAGAG
VEGFR3 (human)	109	56	F: GGTACATGCCAACGACACAG R: CTCAAAGTCTCTCACGAACACG
HPRT (mouse)	163	58.5–62.4	F: GTTGGATACAGGCCAGACTTTGTTG R: GATTCAACTTGCGCTCATCTTAGGC
IRS-1 (mouse)	122	58.5	F: GACGCTCCAGTGAGGATTTAAG R: GGATTTGCTGAGGTCATTTAGG
VEGF-A (mouse)	184	60	F: CATGGATGTCTACCAGCGAAG R: CATGGTGATGTTGCTCTCTGAC
VEGF-C (mouse)	219	60	F: AGAACGTGTCCAAGAAATCAGC R: ATGTGGCCTTTTCCAATACG
VEGF-D (mouse)	86	62.4	F: ATGGCGGCTAGGTGATTCC R: CCCTTCCTTTCTGAGTGCTG
VEGFR3 (mouse)	94	60	F: GTCCCTCTACTTCCAACTGCTTC R: CACTCCTCCTCTGTGACTTTGAG

F, forward; R, reverse.

step of 95°C for 60 seconds and a subsequent melt curve analysis to check amplification specificity. All PCR products were analyzed by gel electrophoresis on a 2% agarose gel and were visualized by ethidium bromide staining. Primer sequences, product sizes, and respective annealing temperatures are summarized in Table 1.

Real-time PCR results were analyzed by the comparative threshold cycle method with human HPRT1 (or mouse HPRT, respectively) as the endogenous reference gene for all reactions. The relative messenger RNA (mRNA) level in the untreated group was used as the normalized control for the treatment groups. All assays were conducted three times and performed in triplicate; a nontemplate control was included in all the experiments to evaluate DNA contamination of the reagents used.

Animals and Anesthesia

All animal protocols were approved by the local animal care committee and were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Mice were anesthetized with intraperitoneal injection of a combination of ketamine (Ketanest S; 8 mg/kg) and xylazine (Rompun; 0.1 mL/kg). All mice were 8- to 10-week-old females (purchased from Charles River Laboratories, Wilmington, MA).

Suture-Induced, Inflammatory Corneal Neovascularization Assay

The mouse model of suture-induced inflammatory corneal suture placement was used as previously described.[19] Before corneal neovascularization, each animal was deeply anesthetized. The healthy cornea actively maintains its avascularity, and, especially, the corneal epithelium contains antiangiogenic molecules such as soluble VEGF receptor 1 and membrane-bound VEGF receptor 3.[23,24] Therefore, to obtain higher angiogenic responses, the central cornea was marked with a 2-mm diameter trephine and deepithelialized before suture placement. Three 11–0 nylon sutures (Serag Wiessner, Naila, Germany) were then placed intrastromally, with two stromal incursions each extending over 120° of corneal circumference. The outer point of suture placement was chosen near the limbus, and the inner suture point was chosen near the corneal center equidistant from the limbus to obtain standardized angiogenic responses. Sutures were left in place for 7 days. Treatment groups received GS-101 eye drops (twice daily for 1 week, 5 μL per drop; 50, 100, or 200 μM). Control mice received equal amounts of saline solution. After 1 week, mice were killed and corneas were prepared. The corneal neovascularization assay included 18 mice per group.

Corneal Wholemounts and Morphologic Determination of Hemangiogenesis and Lymphangiogenesis

The excised corneas from the corneal neovascularization assay were rinsed in PBS and fixed in acetone for 20 minutes. After washing and blocking with 2% bovine serum albumin (BSA) in PBS for 2 hours, corneas were stained overnight at 4°C with a rabbit anti-mouse LYVE-1 antibody (AngioBio, Del Mar, CA) and FITC-conjugated rat anti-mouse CD31 antibody (Acris Antibodies, Herford, Germany). On day 2, LYVE-1 was detected with a Cy3-conjugated secondary goat anti-rabbit antibody (Dianova, Barcelona, Spain). Corneas were moved to slides (Superfrost; Thermo Scientific), covered with fluorescent mounting medium (Dako, Carpinteria, CA) and were stored at 4°C in the dark.

Stained wholemounts were analyzed with a fluorescence microscope (BX51; Olympus Optical Co., Tokyo, Japan), and digital pictures were taken with a 12-bit monochrome CCD camera (F-View II; Soft Imaging System, Münster, Germany). Each wholemount picture was assembled from nine pictures taken at 100× magnification. The area covered with blood and lymphatic vessels was detected with an algorithm established in an image analyzing program (CellF̂; Soft Imaging System). Before analysis, gray value images of the wholemount pictures were modified by several filters, and vessels were detected by threshold setting including the bright vessels and excluding the dark background. A detailed explanation of this method was described previously.[25] The mean vascularized area of the control wholemounts was defined as 100%; vascularized areas were then related to this value (vessel ratio).

Corneal Staining for Macrophages and IRS-1

Naive corneas were deepithelialized, excised, and fixed in acetone for 20 minutes. After washing and blocking with 2% BSA in PBS for 2 hours, corneas were stained overnight at 4°C with a rabbit anti-mouse IRS-1 antibody (Abcam, Cambridge, MA) and FITC-conjugated rat anti-mouse CD11b antibody (Serotec, Raleigh, NC). On day 2, IRS-1 was detected with a Cy3-conjugated secondary goat anti-rabbit antibody (Dianova). Corneas were moved to slides (Superfrost), covered with fluorescent mounting medium (DAKO), and stored at 4°C in the dark.

Statistical Analysis

Statistical analyses were performed (Excel 2000]Microsoft, Redmond, CA] and InStat 3 Version 3.06 [GraphPad Software Inc., San Diego, CA]). Statistical significance was determined using the Student's *t*-test. For the comparison of more than two groups or the analysis of dose-dependent responses, statistical significance was determined using one-way analysis of variance test (ANOVA). $P < 0.05$ was considered statistically significant. Graphs were drawn using biostatistics/curve fitting/scientific graphing software (Prism4, version 4.03; GraphPad Software Inc).

Results

IRS-1 Signaling Blockade by GS-101 Inhibits Lymphatic Endothelial Cell Proliferation

We analyzed the impact of IRS-1 signaling blockade on LEC proliferation in vitro. LECs were exposed to increasing concentrations of GS-101; proliferation was then assessed by ELISA. Incubation with GS-101 significantly suppressed LEC proliferation dose dependently. GS-101 (10 μM) inhibited LEC proliferation by 19% ($P < 0.001$), whereas 20 μM GS-101 led to an inhibition of LEC proliferation by 46% ($P < 0.001$). Higher

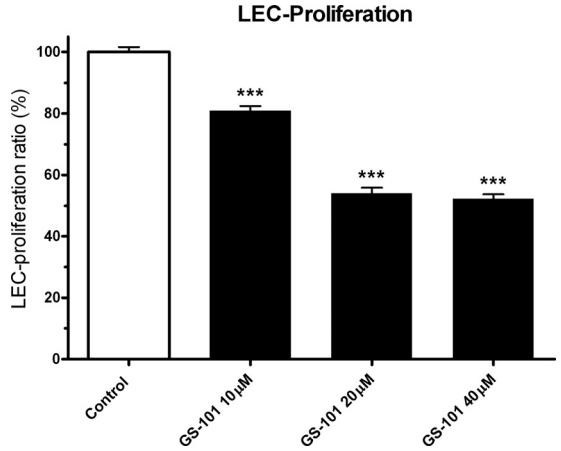

Figure 1. IRS-1 blockade by GS-101 inhibits LEC proliferation in vitro. LECs were exposed to increasing concentrations of GS-101, an antisense oligonucleotide against IRS-1; proliferation was then measured by ELISA. Treatment of LECs with GS-101 significantly suppressed cell proliferation. 10 μM GS-101 inhibited LEC proliferation by 19%, whereas 20 μM GS-101 led to an inhibition of LEC proliferation by 46%. Higher concentrations had no additional inhibitory effect (40 μM GS-101, inhibition by 48%; 20 μM vs. 40 μM; $P > 0.05$; $^{***}P < 0.001$; data are expressed as mean ± SEM).

concentrations had no additional inhibitory effect (40 μM GS-101, inhibition by 48% [$P < 0.001$]; 20 μM vs. 40 μM, not significant [$P > 0.05$]; Fig. 1).

Blockade of IRS-1 in LECs Impairs Expression of VEGF-A but Not of VEGF-C, VEGF-D, or VEGFR3

LECs were incubated with various concentrations of GS-101 for 24 hours. mRNA expression levels of VEGF-A, VEGF-C, VEGF-D, and VEGFR3 were then measured by real-time PCR. VEGF-A expression was inhibited dose dependently: 10 μM GS-101 led to an inhibition of VEGF-A mRNA levels by 14% ($P < 0.05$), and 20 μM inhibited VEGF-A expression by 34% ($P < 0.001$; Fig. 2A). However, VEGF-C and VEGF-D expression levels presented no significant change. VEGF-C expression levels showed a slight, but not yet significant, decrease (10 μM, reduction by 14% [$P = 0.084$]; 20 μM, reduction by 13% [$P = 0.063$]; Fig. 2B), and VEGF-D levels, which were very low, remained unaffected (10 μM, $P = 0.41$; 20 μM, $P = 0.31$; Fig. 2C). VEGFR3 levels also remained unaltered (10 μM, $P = 0.85$; 20 μM, $P = 0.34$; Fig. 2D).

IRS-1 Is Significantly Involved in Pro(lymph)angiogenic Growth Factor Expression by Macrophages

To analyze whether macrophages express IRS-1 and whether IRS-1 is regulated under inflammatory conditions, PECs were incubated in medium alone or with the addition of the proinflammatory cytokines TNF-α or IL-1β. PCR analyses revealed that IRS-1 indeed is expressed in PECs, but the addition of neither TNF-α nor IL-1β led to a considerable change in IRS-1 expression levels. However, the addition of GS-101 significantly suppressed IRS-1 expression in PECs (Fig. 3A).

To analyze the potential involvement of IRS-1 signaling in macrophage-derived growth factor expression, PECs were incubated with GS-101. mRNA expression levels of VEGF-A, VEGF-C, VEGF-D, and VEGFR3 were then measured by real-time PCR. Because our experiments had shown that IRS-1 seems not to be upregulated in PECs under inflammatory conditions, we decided to analyze the effect of IRS-1 blockade only on basal growth factor expression by PECs without previous

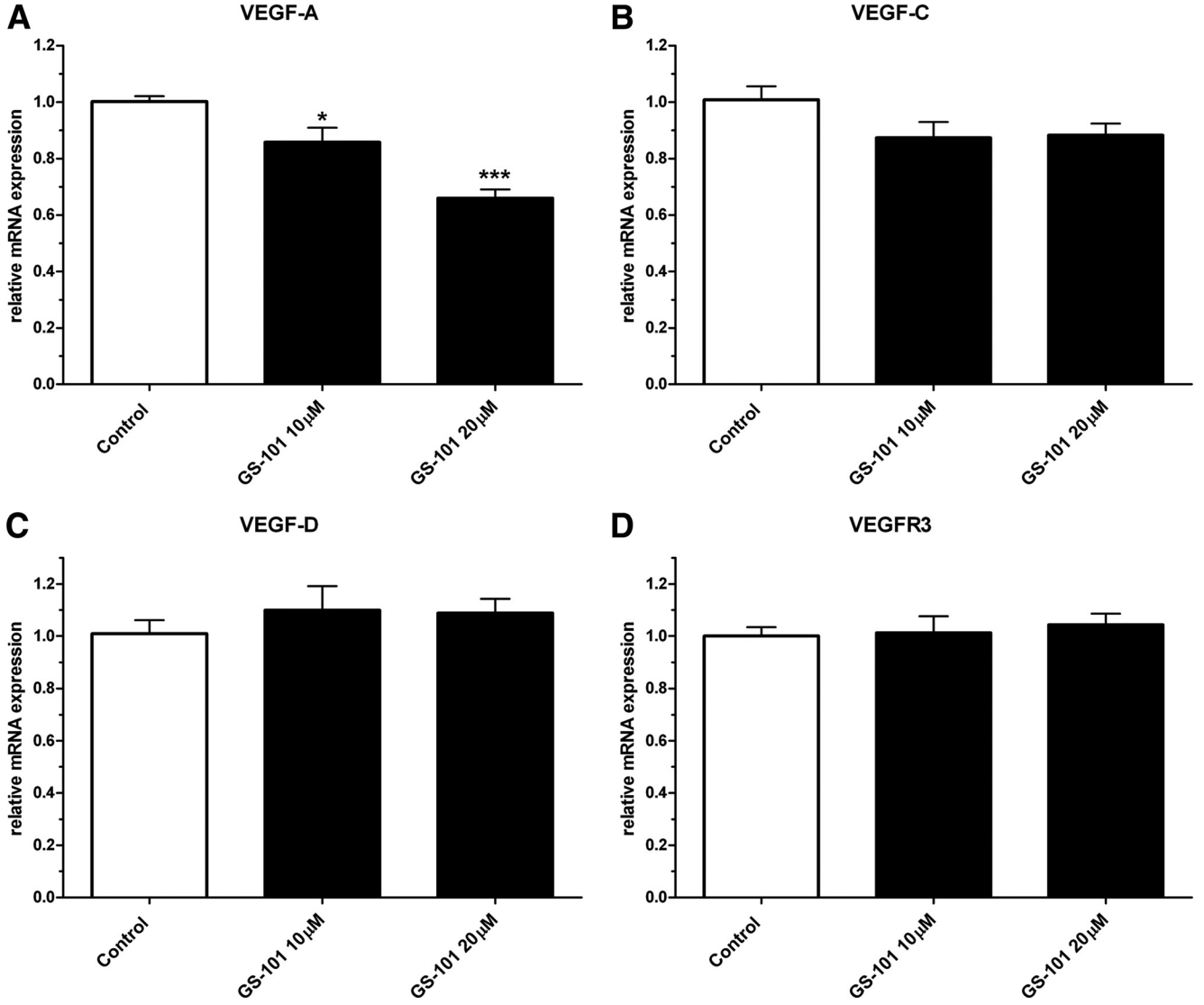

Figure 2. Blockade of IRS-1 in LECs impairs expression of VEGF-A, but not of VEGF-C, VEGF-D, or VEGFR3. LECs were incubated with 10 or 20 μM GS-101. mRNA expression levels of VEGF-A, VEGF-C, VEGF-D, and VEGFR3 were then measured by real-time PCR. (**A**) VEGF-A expression was inhibited dose dependently (10 μM, inhibition by 14%, $P < 0.05$; 20 μM, inhibition by 34%, $P < 0.001$). (**B–D**) VEGF-C, VEGF-D, and VEGFR3 expression levels presented no significant change (all $P > 0.05$). Data are expressed as mean ± SEM. *$P < 0.05$; ***$P < 0.001$.

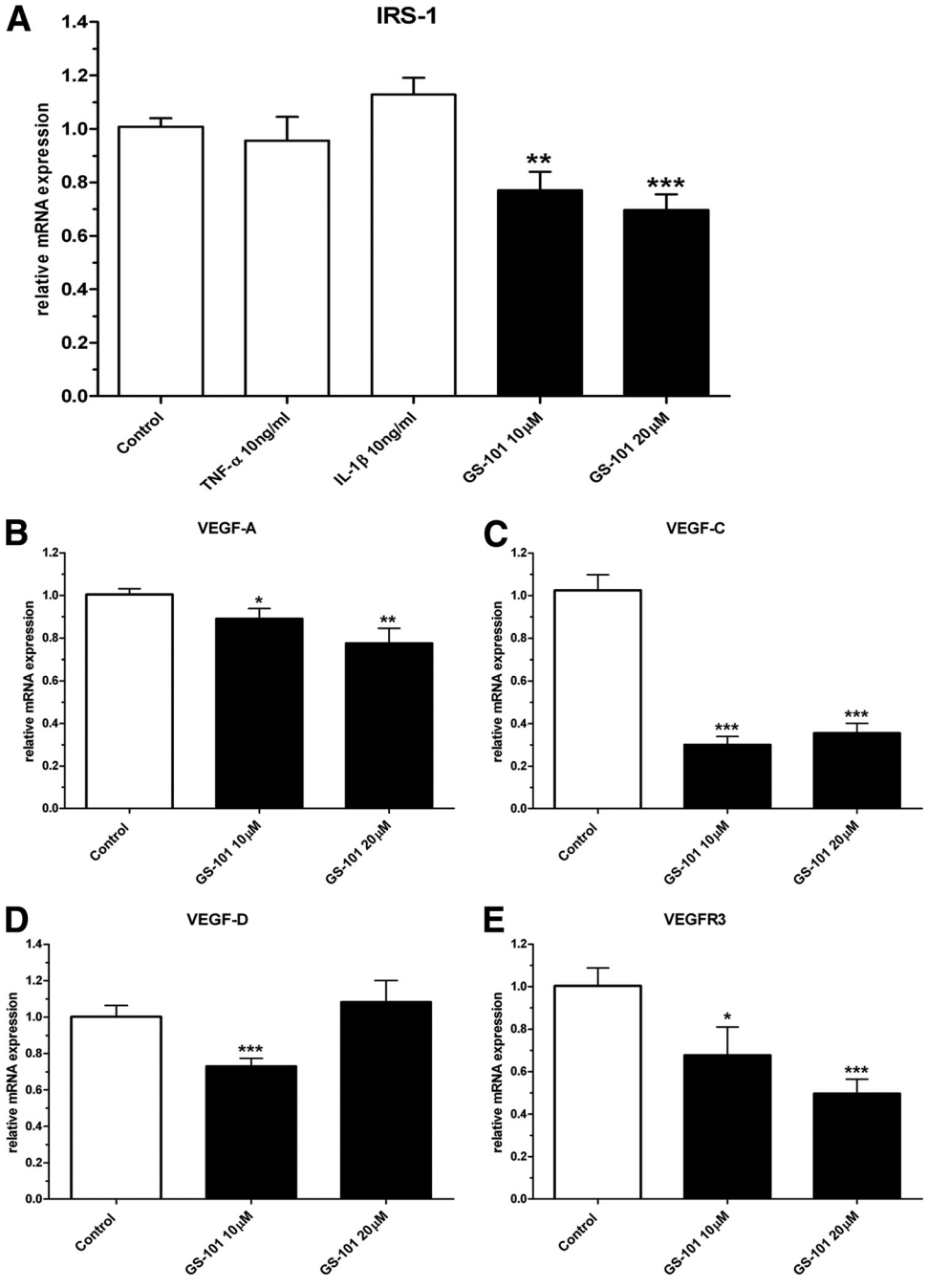

FIGURE 3. Macrophage-derived growth factor expression is reduced after blockade of IRS-1. PECs were incubated with TNF-α, IL-1β, or various concentrations of GS-101. mRNA expression levels of IRS-1, VEGF-A, VEGF-C, VEGF-D and VEGFR3 were then analyzed by real-time PCR. (**A**) TNF-α or IL-1β led to no significant change in IRS-1 expression levels, whereas the addition of GS-101 significantly suppressed IRS-1 expression. (**B–E**) Furthermore, blockade of IRS-1 expression by GS-101 led to a significant downregulation of angiogenic growth factor expression by PECs. Data are expressed as mean ± SEM.

stimulation. Treatment with GS-101 inhibited VEGF-A expression dose dependently: 10 μM GS-101 inhibited VEGF-A mRNA levels by 11% ($P < 0.05$), and 20 μM GS-101 inhibited VEGF-A expression by 23% ($P < 0.01$; Fig. 3B). Notably, VEGF-C expression levels were strongly inhibited by IRS-1 blockade: 10 μM GS-101 led to a decrease in VEGF-C expression levels by 70% ($P < 0.001$), and 20 μM of GS101 inhibited VEGF-C expression by 64% ($P < 0.001$; Fig. 3C). VEGF-D expression was also inhibited, albeit only when GS-101 was used at a dose of 10 μM (reduction by 27%; $P < 0.01$). The inhibition of VEGF-D expression was no longer detectable when GS-101 was used at the higher dose of 20 μM ($P > 0.05$; Fig. 3D). VEGFR3 expression levels also showed a decrease: 10 μM GS-101 led to a reduction of VEGFR3 expression by 33% ($P < 0.05$), and the addition of 20 μM GS-101 led to an inhibition by 50%, $P < 0.001$; Fig. 3E).

Blockade of IRS-1 Signaling Inhibits Hemangiogenesis and Lymphangiogenesis In Vivo

The healthy cornea lacks both blood vessels and lymphatic vessels, but it can secondarily be invaded by both vessel types after severe inflammation. For that reason, the cornea is widely used to study mechanisms of hemangiogenesis and lymphangiogenesis.[26–28] To analyze the value of IRS-1 signaling in vivo, we used the suture-induced corneal neovascularization assay as a well-established and accepted model for induction and analysis of inflammatory corneal hemangiogenesis and lymphangiogenesis.[19,29,30] Blockade of IRS-1 after suture placement led to a significant reduction of inflammatory corneal neovascularization in vivo. When GS-101 was used at the highest dose of 200 μM, corneal hemangiogenesis was significantly inhibited (by

17% in comparison with control animals; $P < 0.01$). Lower concentrations of GS-101 eye drops had no significant effect on blood vessel growth ($P > 0.05$). Corneal lymphangiogenesis was already significantly inhibited by 21% when used at a dose of 100 μM ($P < 0.01$), and the highest dose (200 μM) showed an even stronger inhibition (26% less; $P < 0.001$). When used at the lowest dose of 50 μM, GS-101 eye drops did not show a significant inhibition of corneal lymphangiogenesis ($P > 0.05$; Fig. 4).

Corneal Macrophages Express IRS-1

We stained corneas for CD11b and IRS-1 to analyze whether corneal macrophages also express IRS-1. Figure 5 shows that some, but not all, corneal CD11b$^+$ cells express IRS-1. The expression of IRS-1 by corneal macrophages in vivo, together with the impact of IRS-1 blockade on macrophage-derived growth factor expression in vitro, led us to the conclusion that the inhibition of macrophage-derived IRS-1 expression by GS-101 might indeed be one of the mechanisms contributing to fewer corneal lymphatic vessels in vivo.

Discussion

Research on lymphangiogenesis is an emerging field, and our knowledge of the mechanisms underlying the formation of new lymphatic vessels is steadily expanding. However, the importance of all molecules involved in this process is still not fully understood. Several studies have investigated the role of

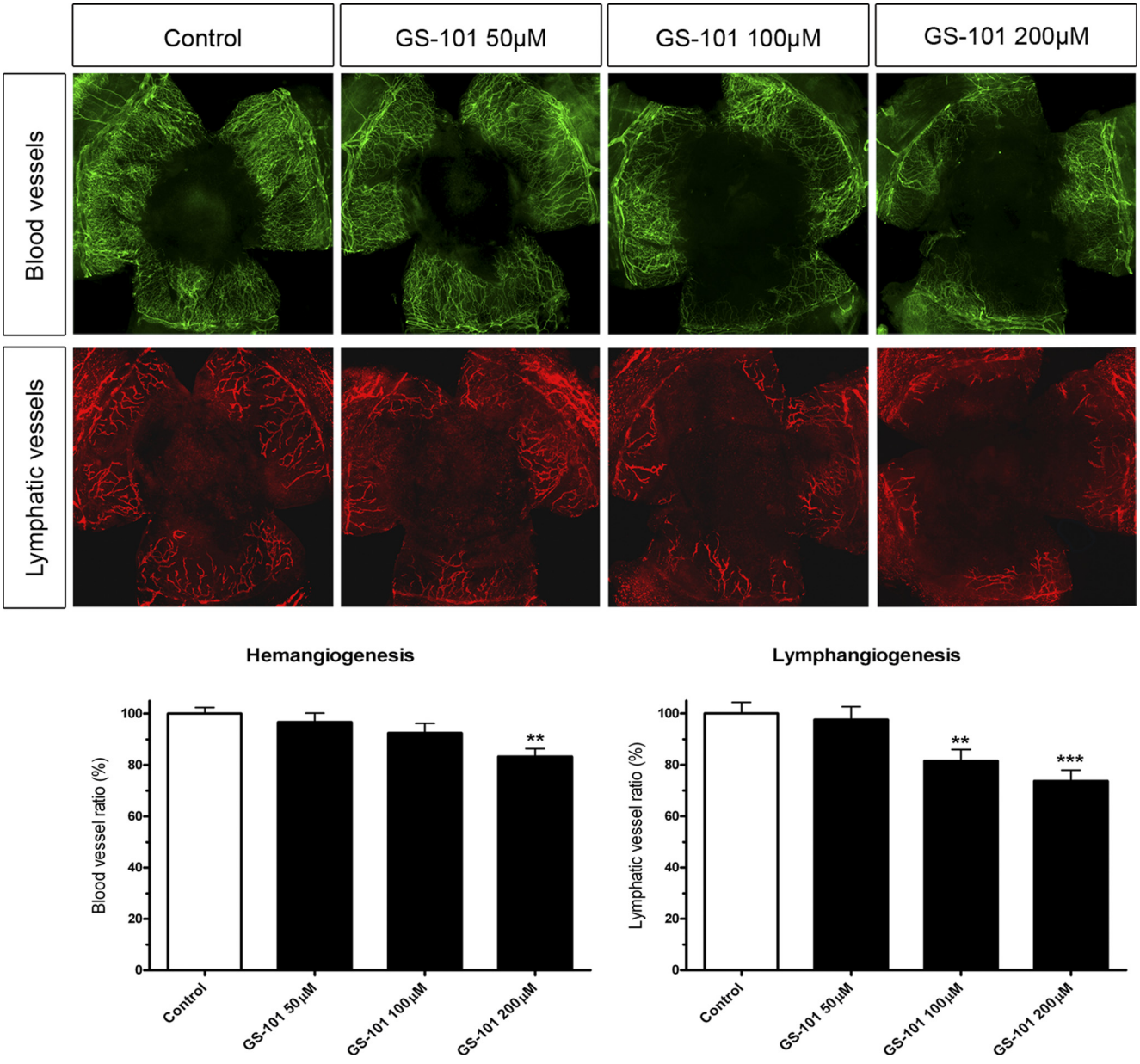

Figure 4. Blockade of IRS-1 leads to reduced corneal hemangiogenesis and lymphangiogenesis in vivo. After suture placement, both corneal hemangiogenesis and lymphangiogenesis were significantly inhibited by topical application of GS-101 eye drops. Corneal hemangiogenesis was significantly inhibited when used at a dose of 200 μM (by 17%; $P < 0.01$). Corneal lymphangiogenesis was significantly inhibited when used at a dose of 100 μM (by 21%; $P < 0.01$), and the highest used dose (200 μM) showed an inhibition of 28% ($P < 0.001$). *Top*: corneal wholemounts stained with CD31 (*green*). *Middle*: corneal wholemounts stained with LYVE-1 (*red*). *Bottom*: quantification of hemangiogenesis and lymphangiogenesis. Data are expressed as mean + SEM. $^{**}P < 0.01$; $^{***}P < 0.001$.

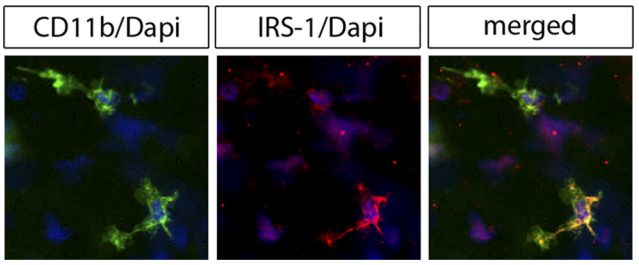

FIGURE 5. Corneal macrophages express IRS-1. Corneal wholemounts stained for IRS-1 and CD11b show a colocalization of IRS-1 and CD11b in some, but not all, $CD11b^+$ cells. *Green*: CD11b-FITC. *Red*: IRS-1-Cy3. *Blue*: DAPI (nuclear staining).

IRS-1 in *hem*angiogenesis,[15–18] but the value of IRS-1 in *lymph*-angiogenesis was thus far not clear. We showed in this study that IRS-1 signaling is also involved in the development of new lymphatic vessels and that the blockade of IRS-1 expression by GS-101 inhibits not only corneal hemangiogenesis but also lymphangiogenesis in vivo.

Inhibition of hemangiogenesis by GS-101 started at a dose of 200 μM, whereas inhibition of lymphangiogenesis started at even lower concentrations, beginning at 100 μM. The significant inhibition of corneal lymphatic vessel growth by GS-101 let us conclude that IRS-1 also has an important role in lymphangiogenesis, with an even stronger impact of its downregulation on lymphatic vessel growth than on blood vessel growth.

It has previously been shown that GS-101 is able to inhibit endothelial tube-like structure formation in human umbilical vein endothelial cells.[17] However, the impact of GS-101 on direct proliferation of lymphatic endothelial cells was thus far not addressed. Blockade of IRS-1 expression by GS-101 inhibited LEC proliferation dose dependently, with maximal inhibition at 20 μM. There was no additional benefit of higher concentrations, which probably indicates a saturation of IRS-1 inhibition. This is in line with previous results showing that 20 μM GS-101 is sufficient to minimize IRS-1 expression in endothelial cells.[17] Additionally, it was previously shown that IRS-$1^{-/-}$ mice develop only 40% less hypoxia-induced retinal neovascularization,[15] demonstrating that IRS-1 is notably involved, but not essential, for angiogenesis.

Furthermore, GS-101 leads to reduced expression levels of VEGF-A and IL-1β in endothelial cells.[17] Besides VEGF-A, we analyzed the effect of GS-101 on the expression levels of VEGF-C, VEGF-D, and VEGFR3 in lymphatic endothelial cells. We could also detect a dose-dependent inhibition of VEGF-A expression. However, neither VEGF-C nor VEGF-D expression was significantly affected by IRS-1 blockade. VEGF-C expression levels showed a slight, but not yet significant, decrease, and VEGF-D levels remained unaffected. This could have been due to different regulatory pathways among the various VEGF members. However, VEGF-A is the VEGF member with the highest expression levels even in *lymphatic* endothelial cells, and it is known that VEGF-D in particular is just barely detectable in endothelial cells.[31] Therefore, we cannot rule out the possibility that we could not identify inhibition caused by already low growth factor levels.

In addition to endothelial cells, macrophages also strongly contribute to lymphangiogenesis.[19,20] It was shown that $CD11b^+$ cells are able to form vessel-like tubes and to integrate into preexisting lymphatic vessels.[20] Furthermore, a multitude of proangiogenic growth factors are secreted by macrophages, leading to a strong augmentation of both hemangiogenesis and lymphangiogenesis.[19] It was previously shown that downregulation of IRS-1 signaling seems to be associated with a decrease in the number of infiltrating macrophages.[16] However, the effect of IRS-1 blockade on pro(lymph)angiogenic factor production by macrophages was not investigated. We could detect a significant downregulation of VEGF-A expression after treatment of macrophages with GS-101. Additionally, VEGF-C expression was strongly suppressed by GS-101, and expression levels of VEGF-D decreased, albeit only after treatment with 10 μM GS-101. Surprisingly, when GS-101 was used at the higher dose of 20 μM, inhibition was no longer detectable. Several studies have reported differential and even paradoxical regulations of the various VEGF members. Moffat et al.[32] demonstrated that tumor cells underexpressing VEGF-A showed higher levels of VEGF-D. On the other hand, O-charoenrat et al.[33] showed that several growth factors that upregulate VEGF-A lead to a downregulation of VEGF-D expression levels. This could also be in line with our results: high doses of GS-101 might reduce VEGF-A expression below a certain threshold, which then possibly antagonizes the (direct) impact of GS-101 on VEGF-D and, therefore, leads to a subsequent loss of inhibition. Certainly, further investigation is needed to provide evidence for this rather speculative hypothesis.

Altogether, we conclude that IRS-1 blockade seems to suppress a variety of processes leading to the development of new lymphatic vessels. One of the early steps in GS-101 action appears to be inhibition of the number of infiltrating macrophages, as described previously.[16] It is known that macrophages promote lymphangiogenesis in two different ways, either by stimulating preexistent lymphatic endothelial cells or by transdifferentiating and directly forming new lymphatic vessels. This decisive role of infiltrating macrophages, especially in the development of lymphatic vessels, could be a possible explanation of the earlier inhibition of lymphangiogenesis (starting at a dose of 100 μM) rather than of hemangiogenesis (starting at a dose of 200 μM) observed in our in vivo experiments. It is also known that GS-101 diminishes the overall expression of several angiogenic growth factors in the cornea.[16] Moreover, as shown in our study, the quantity of growth factors expressed per macrophage also decreases after IRS-1 blockade. This might be another explanation for a stronger inhibition of lymphangiogenesis given that macrophages are known to secrete several factors specific for lymphangiogenesis but not for hemangiogenesis, namely VEGF-C and VEGF-D, whereas most of the factors leading to blood vessel growth also promote lymphatic vessel growth, such as VEGF-A. Additionally, IRS-1 directly impairs endothelial cell function. It is the task of further investigation to analyze whether lymphatic endothelial cells are more susceptible than blood endothelial cells to IRS-1 blockade.

In summary, we have shown that the blockade of IRS-1 expression by GS-101 inhibits not only corneal hemangiogenesis but also lymphangiogenesis. The effects of GS-101 action seem to occur through its direct interaction with lymphatic endothelial cells, namely proliferation inhibition and VEGF-A expression. Furthermore, IRS-1 blockade impairs lymphangiogenesis indirectly by reducing macrophage-derived growth factor expression (VEGF-A and, especially, VEGF-C). This is, to our knowledge, the first evidence that IRS-1 signaling is involved in the molecular pathway leading to lymphangiogenesis.

Acknowledgments

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This Month in *AJP*

Periostin and Exocrine Pancreatic Tissue

Despite known functions of the extracellular matrix molecule periostin in chronic pancreatitis and pancreatic cancer, its role in acute pancreatitis remains unclear. Hausmann et al (**Am J Pathol 2016, 186:24–31**) studied its function in pancreatic exocrine regeneration following severe acute pancreatitis (AP). Severe AP was induced in adult mice with and without global periostin ablation. Detailed histological analysis revealed similar pancreatitis severity in the acute inflammatory phase in all mice. However, in periostin-deficient mice the recovery of the exocrine pancreas was vastly impaired, and acinar-to-adipocyte differentiation as well as expression levels of pancreatic and acinar differentiation markers were disturbed. Periostin regulates acinar cell fate decision and restores pancreatic tissue integrity following AP.

Granzyme B Mediates Cardiac Fibrosis

The serine protease Granzyme B (GzmB) contributes to several fibrosis-related cellular processes, but a direct link with cardiac fibrosis is missing. Using fibrotic human hearts and an established angiotensin II (Ang II)–induced cardiac fibrosis mouse model, Shen et al (**Am J Pathol 2016, 186:87–100**) studied the role of GzmB in the pathogenesis of cardiac fibrosis. GzmB was up-regulated in both fibrotic human and murine hearts. In mice, GzmB deficiency protected against Ang II–induced cardiac hypertrophy and cardiac fibrosis—independent of perforin—by reducing microhemorrhage, inflammation, and fibroblast accumulation. *In vitro*, GzmB directly cleaved the endothelial junction protein VE-cadherin, disrupting barrier function. Targeting extracellular GzmB may halt the progression of cardiac fibrosis.

Mast Cell–Derived Histamine Promotes Cholangiocarcinoma

Mast cells (MCs) contribute to the pathogenesis of cholangiocarcinoma (CCA) by releasing inflammatory factors that support tumor progression. Using *in vitro* and *in vivo* models, Johnson et al (**Am J Pathol 2016, 186:123–133**) dissected the role of MCs in the pathophysiology of CCA. MC infiltration into the CCA microenvironment and the expression of MC markers were observed in human biopsies and mouse tumors. Blocking MC-derived histamine decreased tumor growth, proliferation, angiogenesis, epithelial-mesenchymal transition, and extracellular matrix degradation via inhibition of c-Kit/stem cell factor. Preventing MC migration may be an important target for CCA therapy.

IL-10 Regulates Inflammatory Corneal Lymphangiogenesis

How the anti-inflammatory cytokine IL-10 regulates inflammatory lymphangiogenesis is unknown. Using an established mouse model, Hos et al (**Am J Pathol 2016, 186:159–171**) determined the impact of IL-10 on inflammatory corneal lymphangiogenesis and the resolution of corneal inflammation. IL-10 was detected in inflamed, but not healthy, corneas and was expressed by infiltrating macrophages. *In vitro* IL-10 stimulation up-regulated the expression of pro-lymphangiogenic vascular endothelial growth factor-C in macrophages but did not affect lymphatic endothelial cells. *In vivo* IL-10 deficiency reduced corneal lymphangiogenesis and prolonged corneal inflammation. Local treatment with IL-10 promoted lymphangiogenesis and faster egress of macrophages from inflamed corneas. IL-10 may be useful therapeutically to resolve pathological inflammation in the cornea.

Aβ Precedes p-Tau in Alzheimer Disease Synapses

The detailed time course of amyloid-β (Aβ) and hyperphosphorylated tau (p-tau) accumulation in Alzheimer disease (AD) patient synapses is unclear. Bilousova et al (**Am J Pathol 2016, 186:185–198**) investigated this sequence using patient samples and a transgenic rat model. Aβ and p-tau were quantified across AD disease stages (including non-demented high AD–related pathology controls) in parietal cortex. Aβ accumulated in the earliest plaque stages as well as in late-stage AD dementia whereas p-tau appeared essentially in late-stage disease. Synapse-associated soluble oligomers of Aβ were linked to the onset of dementia. p-tau was elevated in individual Aβ-positive synaptosomes in early AD, arguing for an amyloid cascade hypothesis driving p-tau accumulation.

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GROWTH FACTORS, CYTOKINES, AND CELL CYCLE MOLECULES

IL-10 Indirectly Regulates Corneal Lymphangiogenesis and Resolution of Inflammation via Macrophages

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The role of IL-10, a primarily anti-inflammatory cytokine, in the regulation of inflammatory lymphangiogenesis is undetermined. Herein, we show that IL-10 modulates corneal lymphangiogenesis and resolution of inflammation. IL-10 was not expressed in healthy corneas but was up-regulated in inflamed corneas by infiltrating macrophages. Macrophages up-regulated the expression of prolymphangiogenic vascular endothelial growth factor-C upon stimulation with IL-10. Consistently, corneal inflammation resulted in reduced expression of vascular endothelial growth factor-C and decreased corneal lymphangiogenesis in IL-10—deficient mice ($IL\text{-}10^{-/-}$). The effect of IL-10 on lymphangiogenesis was indirect via macrophages, because IL-10 did not directly affect lymphatic endothelial cells. The expression of proinflammatory cytokines and the numbers of infiltrating macrophages increased and remained elevated in inflamed corneas of $IL\text{-}10^{-/-}$ mice, indicating that IL-10 deficiency led to more severe and prolonged inflammation. The corneal phenotype of IL-10 deficient mice was mimicked in mice with conditional deletion of Stat3 in myeloid cells (lysozyme M Cre mice $Stat3^{fl/fl}$ mice), corroborating the critical role of macrophages in the regulation of lymphangiogenesis. Furthermore, local treatment with IL-10 promoted lymphangiogenesis and faster egress of macrophages from inflamed corneas. Taken together, we demonstrate that IL-10 indirectly regulates inflammatory corneal lymphangiogenesis via macrophages. Reduced lymphangiogenesis in $IL\text{-}10^{-/-}$ and lysozyme M Cre $Stat3^{fl/fl}$ mice is associated with more severe inflammatory responses, whereas IL-10 treatment results in faster resolution of inflammation. IL-10 might be used therapeutically to terminate pathological inflammation. *(Am J Pathol 2016, 186: 159—171; http://dx.doi.org/10.1016/j.ajpath.2015.09.012)*

The lymphatic vasculature, also termed the second vascular system, is involved in fundamental physiological functions, such as fluid and lipid homeostasis, blood pressure regulation, inflammation, and immune surveillance.[1,2] Moreover, dysregulation of the lymphatic vascular system also contributes to several pathological conditions, such as lymphedema, cancer metastasis, transplant rejection, and chronic wounds.[2,3] Dissecting the cellular and molecular mechanisms that regulate lymphangiogenesis in physiology and pathology is therefore crucial for the development of efficient therapeutic approaches to prevent disease and to promote cure.

The cornea, which is the transparent windscreen of the eye, is one of the rare tissues of the organism that is physiologically devoid of lymphatic vessels.[4,5] Corneal avascularity is not a passive condition but is actively maintained by several redundantly organized anti(lymph) angiogenic mechanisms.[6—10] Minor inflammatory stimuli, to which the cornea is continuously exposed because of its anatomical position, usually do not induce corneal lymphangiogenesis. Severe inflammation, however, can result

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in a strong up-regulation of prolymphangiogenic growth factors that overcome the cornea's antilymphangiogenic barriers and result in a secondary ingrowth of lymphatic vessels into the cornea.[5] In this context, macrophages have been identified as essential mediators of inflammatory corneal lymphangiogenesis. It is well established that macrophages are able to secrete a variety of prolymphangiogenic growth factors, such as vascular endothelial growth factor (VEGF)-C and VEGF-D, thereby inducing lymphatic endothelial cell (LEC) proliferation.[11,12] Various functional macrophage phenotypes exist, which have, for a long time, been classified into at least two polarized macrophage subpopulations[13,14]: classically activated, M1-polarized macrophages are considered to exert proinflammatory activities, eradicate invading microorganisms, and promote type I immune responses. Alternatively activated, M2-polarized macrophages are hyporesponsive to proinflammatory stimuli, and are involved in debris scavenging, tissue remodeling, and the resolution of inflammatory responses.[13,15,16] However, in view of recent research, it has become clear that the paradigm of M1/M2 polarization is an oversimplification that reflects only two extremes of macrophage polarization and that in tissues a broad spectrum of activation states exists in parallel.[17,18] Although it is known that macrophages contribute to corneal lymphangiogenesis,[11,12] the underlying mechanisms and especially the involvement of specific macrophage subpopulations remain to be elucidated.

Recently, it has been demonstrated that corneal lymphatic vessels play a critical role in the induction and maintenance of various inflammatory diseases at the ocular surface, such as dry eye disease, ocular allergy, and immune-mediated graft rejection after corneal transplantation.[19–21] Thus, corneal lymphangiogenesis is mostly considered as pathological. In contrast, physiological functions for lymphatic vessels in the cornea have not been described so far. However, studies in extraocular tissues (eg, the skin) demonstrate that lymphatic vessels also exert physiological functions during inflammatory reactions, because lymphatic vessels regulate tissue pressure and allow the drainage of debris and egress of cells from the inflamed site.[22] In particular, lymphatic vessels seem to be important for the termination of ongoing inflammatory responses, and recent studies indicate that the blockade of lymphatic vessels can result in chronic inflammation and edema formation, whereas the specific activation of lymphatic vessels can ameliorate these conditions.[23,24] Thus, lymphangiogenesis and inflammation are closely linked. Furthermore, both processes appear to be, at least partially, regulated by the same molecules and cytokines.[9,11,25–27]

IL-10 is a multifunctional cytokine that is well-known for its anti-inflammatory and immunoregulatory effects.[28] Generally, IL-10 is considered to inhibit immune responses, but under certain circumstances may also have activating properties (eg, on macrophages).[13,29] In addition to its wide-ranging immune-modulatory functions, IL-10 also regulates hemangiogenesis, the development of blood vessels. Several studies demonstrate that IL-10 has antihemangiogenic properties,[30,31] whereas other studies indicate a prohemangiogenic effect of IL-10.[32,33]

In contrast to previous studies addressing the impact of IL-10 on hemangiogenesis,[30–33] no single study has analyzed the role of IL-10 in lymphangiogenesis so far. We therefore used the cornea as a well-established model system to study lymphangiogenesis and determined the impact of IL-10 on inflammatory corneal lymphangiogenesis and the resolution of corneal inflammation. Our results provide evidence that IL-10 regulates corneal lymphangiogenesis, and that its effect is possibly indirectly mediated through the expression of VEGF-C by macrophages. Furthermore, our experiments demonstrate a crucial role for IL-10 in the termination of inflammatory responses at the cornea.

Materials and Methods

Animals

All animal protocols were approved by the local animal care and use committee and were in accordance with the Association for Research in Vision and Ophthalmology's Statement for the Use of Animals in Ophthalmology and Vision Research. The following 8- to 12-week-old mice were used: C57BL/6 wild-type mice (WT), IL-10 homozygous knockout mice (IL-10$^{-/-}$), and lysozyme M Cre mice (LysMCre) crossed with loxP-flanked Stat 3 mice (Stat3$^{fl/fl}$).[34,35] Genotyping of IL-10 mice was performed using the following primers: 5′-CTTGCACTACCAAAGCCACA-3′, 5′-GTTATTGTCTTCCCGGCTGT-3′, and 5′-CCACACGCGTCACCTTAATA-3′. Product sizes of IL-10 WT and knockout alleles were 137 and 312 bp, respectively. The following primers were used for genotyping of LysMCre mice: 5′-CTTGGGCTGCCAGAATTTCTC-3′, 5′-TTACAGTCGGCCAGGCTGAC-3′, and 5′-CCCAGAAATGCCAGATTACG-3′. Product sizes of LysM WT and LysMCre alleles were 350 and 700 bp, respectively. Genotyping of Stat3$^{fl/fl}$ mice was performed using the following primers: 5′-CCTGAAGACCAAGTTCATCTGTGTGAC-3′ and 5′-CACACAAGCCATCAAACTCTGGTCTCC-3′. Product sizes of the Stat3 WT and floxed Stat3 alleles were 200 and 350 bp, respectively.

Mouse Model of Suture-Induced Corneal Inflammation and Neovascularization

The mouse model of suture-induced corneal inflammation and neovascularization was performed as previously described.[11,36,37] Before surgery, mice were anesthetized with an i.p. injection of a combination of ketamine and xylazine hydrochloride. Three 11-0 nylon sutures (Serag Wiessner, Naila, Germany) were then placed intrastromally into the right cornea, with two incursions each extending over 120° of the total corneal circumference. The outer point of suture placement was chosen near the limbus, and the inner suture point was chosen near the corneal center. The

suture model is a well-established procedure known to result in severe corneal inflammation with recruitment of inflammatory cells and subsequent development of corneal blood and lymphatic vessels, which peaks after 2 weeks.[37,38] Sutures are usually removed after 2 weeks, and inflammation and blood and lymphatic vessels then begin to decline.[38] In indicated experiments, mice were treated with repeated subconjunctival injections of recombinant murine IL-10 [100 ng in 10 μL of phosphate-buffered saline (PBS), every other day; R&D Systems, Wiesbaden, Germany] after suture removal for 1 week. Control mice received equal amounts of PBS.

mRNA Isolation and Real-Time PCR

Total RNA from murine corneas (three to five corneas per analyzed time point, excised without the limbus), peritoneal exudate cells (PECs), or LECs were isolated by RNeasy Micro Kit (Qiagen, Valencia, CA), and cDNA (500 ng per group) was synthesized with random hexamers using reverse transcriptase (SuperScript III; Invitrogen, Carlsbad, CA). Primers (MWG Biotech, Ebersberg, Germany) were designed using Primer3 software version 4.0.0 and Basic Local Alignment Search Tool (National Center for Biotechnology Information, Bethesda, MD). PCR reactions (25 μL) contained 50 ng of cDNA, 0.4 μmol/L of each forward and reverse primer, and master mix (SsoFast EvaGreen Supermix; Bio-Rad, Hercules, CA). Real-time PCR was performed under the following conditions: initial denaturation step of 95°C for 2 minutes, 40 cycles of 95°C for 5 seconds and 60°C to 63°C (depending on the analyzed gene) for 15 seconds, followed by an additional denaturation step of 95°C for 60 seconds and a subsequent melt curve analysis to check amplification specificity. All PCR products were analyzed by gel electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining. Real-time PCR results were analyzed by the comparative threshold cycle method with hypoxanthine-guanine phosphoribosyltransferase (HPRT) (mouse)/HPRT1 (human) as the endogenous reference gene for all reactions. The relative mRNA level of WT/PBS-treated corneas or untreated PECs/LECs was used as normalized control. All assays were performed in triplicate, and a nontemplate control was included in all experiments to exclude DNA contamination. Primer sequences, product size, and respective annealing temperatures are summarized in Table 1.

Corneal Immunohistochemistry

Suture placement was performed in WT, and sutures were left in place for 2 weeks. Eyes were then enucleated and embedded

Table 1 Primer Used for Real-Time PCR

mRNA	Product size (bp)	Annealing temperature (°C)	Sequence
HPRT (mouse)	163	60—63	F: 5′-GTTGGATACAGGCCAGACTTTGTTG-3′
			R: 5′-GATTCAACTTGCGCTCATCTTAGGC-3′
TNF-α (mouse)	87	63	F: 5′-AGGACTCAAATGGGCTTTCC-3′
			R: 5′-CAGAGGCAACCTGACCACTC-3′
IL-1β (mouse)	176	62.4	F: 5′-GTCCTGTGTAATGAAAGACGGC-3′
			R: 5′-CTGCTTGTGAGGTGCTGATGTA-3′
IL-10 (mouse)	151	63	F: 5′-CAGTACAGCCGGGAAGACAATA-3′
			R: 5′-GCATTAAGGAGTCGGTTAGCAG-3′
Arginase-1 (mouse)	126	60	F: 5′-GCAGAGGTCCAGAAGAATGG-3′
			R: 5′-GTGAGCATCCACCCAAATG-3′
CD163 (mouse)	153	60	F: 5′-GGCACTCTTGGTTTGTGGAG-3′
			R: 5′-GCCTTTGAATCCATCTCTTGG-3′
LYVE-1 (mouse)	198	60	F: 5′-GGAAGAATGGCAAAGGTGTC-3′
			R: 5′-CAGGGGATGAAGCCAAGTAG-3′
VEGF-A (mouse)	184	60	F: 5′-CATGGATGTCTACCAGCGAAG-3′
			R: 5′-CATGGTGATGTTGCTCTCTGAC-3′
VEGF-C (mouse)	219	60	F: 5′-AGAACGTGTCCAAGAAATCAGC-3′
			R: 5′-ATGTGGCCTTTTCCAATACG-3′
VEGF-D (mouse)	86	62.4	F: 5′-ATGGCGGCTAGGTGATTCC-3′
			R: 5′-CCCTTCCTTTCTGAGTGCTG-3′
HPRT1 (human)	162	60	F: 5′-CCTGGCGTCGTGATTAGTG-3′
			R: 5′-GCCTCCCATCTCCTTCATC-3′
VEGF-C (human)	163	60	F: 5′-GCCTGTGAATGTACAGAAAGTCC-3′
			R: 5′-AATATGAAGGGACACAACGACAC-3′
LYVE-1 (human)	138	60	F: 5′-GCTTGCTCTCCTCTTCTTTGG-3′
			R: 5′-GCCTTCTCCTCCTTTACTACTTTG-3′

F, forward; HPRT, hypoxanthine-guanine phosphoribosyltransferase; LYVE, lymphatic vessel endothelial hyaluronan receptor; R, reverse; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

in OCT Tissue Tek Compound and cryosectioned (8 μm thick). Cryosections were fixed with acetone, blocked in 2% bovine serum albumin in PBS, and stained overnight with the respective primary antibodies. Used primary antibodies were as follows: unconjugated anti–IL-10 (Abcam, Cambridge, UK), fluorescein isothiocyanate–conjugated anti-CD11b (Serotec, Raleigh, NC), unconjugated anti–VEGF-C (Novus Biologicals, Abingdon, UK), or unconjugated anti–lymphatic vessel endothelial hyaluronan receptor (LYVE)-1 (AngioBio, Del Mar, CA). On the next day, (unconjugated) primary antibodies were detected with Cy3-conjugated (Dianova, Barcelona, Spain) or Alexa 488–conjugated (Life Technologies, Carlsbad, CA) secondary antibodies. DAPI was used for nuclear counterstaining. Images were taken with a fluorescence microscope (Olympus BX53; Olympus, Hamburg, Germany).

Generation and Culture of Peritoneal Macrophages

Thioglycollate-induced PECs were collected from the peritoneal cavity of WT or LysMCre Stat3$^{fl/fl}$ 3 days after 2 mL i.p. thioglycollate injection, as previously described.[36,39] PECs were washed, resuspended, and cultured at 37°C in RPMI 1640 medium containing 10% fetal calf serum, 10 mmol/L HEPES, 1 mmol/L nonessential amino acids, 1 mmol/L sodium pyruvate, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin. After adhesion, nonadherent cells were removed by washing with culture medium, and adherent cells were then used as macrophages. Cells collected by this method are F4/80$^+$ (>90%) and CD11b$^+$ (>99%).[12,40] For RNA expression analyses, cells were incubated in RPMI 1640 medium containing 100 ng/mL recombinant murine IL-10 (R&D Systems) for 24 hours, followed by RNA isolation and real-time PCR.

LEC Culture and Proliferation ELISA

Human adult LECs (PromoCell GmbH, Heidelberg, Germany) were cultured in EGM2-MV medium containing 5% fetal calf serum. For cell proliferation analysis, a 5-bromo-2′-deoxyuridine–based proliferation enzyme-linked immunosorbent assay (ELISA) was used, as previously described.[36,39,41] Briefly, cells were seeded in a 96-well plate at a density of 4×10^3 cells per well ($n = 6$ wells per group) and cultured overnight before medium was replaced with EGM2-MV medium, 5-bromo-2′-deoxyuridine, 100 ng/mL VEGF-C, 100 ng/mL IL-10, or a combination of both. After 48 hours, cells were fixed and stained according to the manufacturer's instructions. Colorimetric analysis was performed with an ELISA reader (Epoch Microplate Spectrophotometer; BioTek, Bad Friedrichshall, Germany). The mean extinction of the control wells was defined as 100%, and the extinction of all wells was then related to this value (proliferation index). The experiment was performed twice.

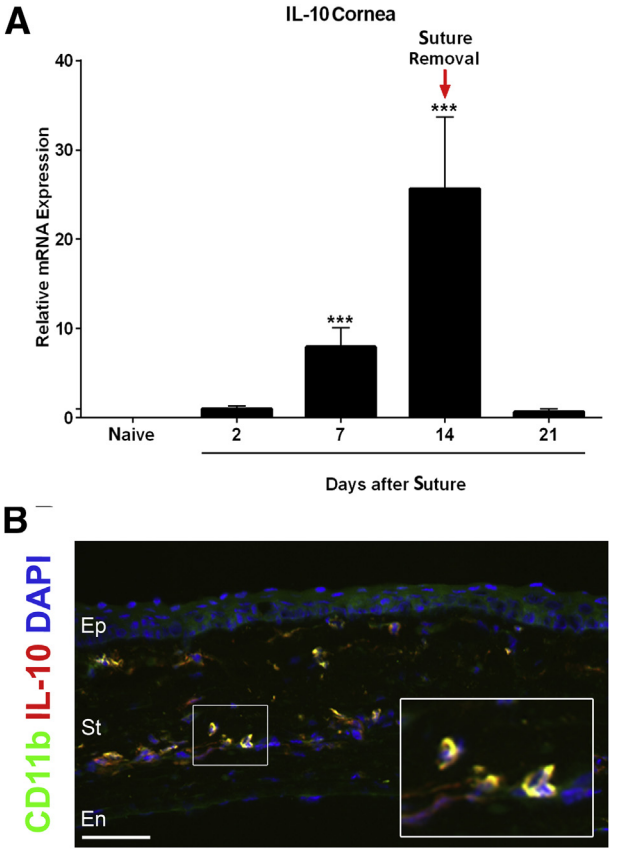

Figure 1 IL-10 expression is up-regulated in CD11b$^+$ cells during corneal inflammation. **A:** IL-10 mRNA expression after corneal suture placement. IL-10 mRNA is not detectable in naive, but only in inflamed, corneas (IL-10 mRNA level on day 2 after suture placement was chosen as the normalized control for following time points). IL-10 expression decreases after suture removal (2 weeks after placement; **arrow**). **B:** IL-10 protein expression in corneal CD11b$^+$ cells. Cryosection of inflamed corneas (2 weeks after suture placement) shows colocalization of CD11b (green) and IL-10 (red). Blue, DAPI nuclear staining. ***$P < 0.001$. Scale bar = 40 μm (**B**). En, corneal endothelium; Ep, corneal epithelium; St, corneal stroma.

VEGF-C ELISA

Total protein from naive and inflamed corneas of WT and IL-10$^{-/-}$ mice (four corneas per group, excised without the limbus) was isolated by Qproteome Mammalian Protein Prep Kit (Qiagen, Valencia, CA). Protein concentrations were determined with Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Schwerte, Germany). VEGF-C ELISA was subsequently performed in triplicates with a mouse VEGF-C ELISA Kit (USCN Life Science Inc., Wuhan, China), according to the manufacturer's recommendations.

Analysis of Inflammatory Corneal Hemangiogenesis and Lymphangiogenesis

For the assessment of inflammatory corneal hemangiogenesis and lymphangiogenesis, corneal suture placement was performed in WT, IL-10$^{-/-}$, Stat3$^{fl/fl}$, LysMCre Stat3$^{fl/fl}$,

and WT treated with recombinant murine IL-10 or PBS. After the indicated time points, corneas ($n = 5$ to 10 per group) were excised, fixed in acetone, blocked with 2% bovine serum albumin in PBS, and stained overnight with a LYVE-1 antibody (AngioBio, Del Mar, CA) and in indicated experiments with a fluorescein isothiocyanate–conjugated CD31 antibody (Acris Antibodies, Herford, Germany). On the next day, LYVE-1 was detected with a Cy3-conjugated secondary antibody (Dianova). Whole mount images were assembled automatically from 9 to 12 images taken at ×100 magnification with a fluorescence microscope (Olympus BX53). Afterward, the area covered with blood and lymphatic vessels was detected with an algorithm established in the image analyzing program Cell^F (Olympus Soft Imaging Solutions GmbH, Münster, Germany), as previously described.[42] Briefly, before analysis, gray value images of the whole mount images were modified by several filters, and vessels were then detected by threshold setting, including the bright vessels and excluding the dark background. The mean vascularized area of control corneas was defined as 100%, and vascularized areas were then related to this value.

Analysis of Corneal Immune Cell Infiltration

For the assessment of immune cell infiltration into the inflamed cornea, corneal suture placement was performed in WT, IL-$10^{-/-}$, Stat3$^{fl/fl}$, LysMCre Stat3$^{fl/fl}$, and WT treated with recombinant murine IL-10 or PBS. After the indicated time points, corneas ($n = 5$ per group) were excised and fixed in acetone, blocked with 2% bovine serum albumin in PBS, and stained overnight at 4°C with a fluorescein isothiocyanate–conjugated CD11b antibody (Serotec). Images of the central cornea were taken at ×200 magnification with focus on the subepithelial corneal layer (Olympus BX53), and CD11b^{+} cells were then counted.

Statistical Analysis

Statistical analyses were performed with Microsoft Excel 2010 (Microsoft Corp., Redmond, WA) and InStat version 3.06 (GraphPad Software Inc., San Diego, CA). Statistical significance was determined using the t-test. $P < 0.05$ was considered statistically significant. Graphs were drawn using Prism4 version 4.03 (GraphPad Software Inc.).

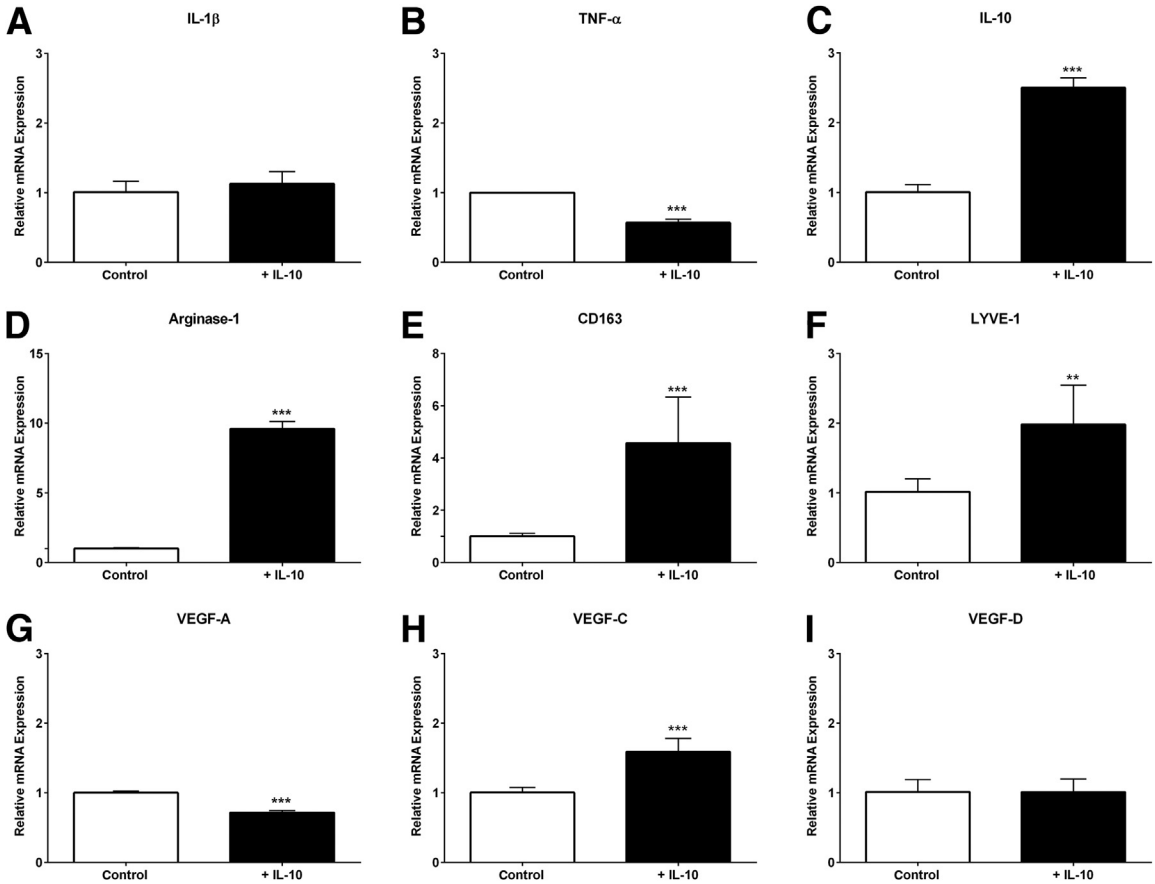

Figure 2 Gene expression pattern of macrophages after IL-10 stimulation indicates anti-inflammatory and prolymphangiogenic phenotype and autocrine amplification cascade. Thioglycollate-activated peritoneal macrophages were incubated with 100 ng/mL IL-10. After 24 hours, mRNA expression of IL-1β (**A**), tumor necrosis factor (TNF)-α (**B**), IL-10 (**C**), arginase-1 (**D**), CD163 (**E**), lymphatic vessel endothelial hyaluronan receptor (LYVE)-1 (**F**), vascular endothelial growth factor (VEGF)-A (**G**), VEGF-C (**H**), and VEGF-D (**I**) was analyzed. IL-1β expression remains unaltered, whereas TNF-α expression is significantly reduced by IL-10. Expression levels of IL-10, arginase-1, CD163, and LYVE-1 are significantly up-regulated after stimulation with IL-10. VEGF-A expression decreases, whereas VEGF-C expression is significantly up-regulated by IL-10. VEGF-D expression remains unchanged. $^{**}P < 0.01$, $^{***}P < 0.001$.

Results

IL-10 Expression Is Up-Regulated in CD11b$^+$ Cells during Corneal Inflammation

It is unclear whether IL-10 is involved in inflammatory responses at the cornea. We therefore analyzed the expression of IL-10 in healthy and inflamed corneas after corneal suture placement in mice, which is a well-defined and established model to induce sterile corneal inflammation dominated by the influx of macrophages.[12,37,39] In healthy corneas, IL-10 mRNA was not measurable. However, 2 days after suture placement, IL-10 mRNA was induced in inflamed corneas and was further up-regulated during the time course of the inflammatory response (1 week: $\times 9.1$, $P < 0.001$; 2 weeks: $\times 29.5$, $P < 0.001$) (Figure 1A). After suture removal (2 weeks after placement), IL-10 mRNA expression declined and was comparable to uninflamed corneas (1 week after suture removal: $\times 0.7$) (Figure 1A). To further verify IL-10 expression on protein level and to identify the IL-10–expressing cell type, we additionally performed immunohistochemistry on corneal sections obtained after suture placement. IL-10 protein expression colocalized with CD11b, which is mainly expressed by leukocytes, such as macrophages (Figure 1B). Thus, IL-10 is absent in healthy corneas, but is highly expressed during corneal inflammation in infiltrating macrophages.

Gene Expression Pattern of Macrophages after IL-10 Stimulation Indicates Anti-Inflammatory and Prolymphangiogenic Phenotype and Autocrine Amplification Cascade

We hypothesized that IL-10 expressed by macrophages might have an autocrine effect and therefore determined the impact of IL-10 on peritoneal macrophages (PECs) *in vitro*. Stimulation of PECs with recombinant IL-10 did not affect IL-1β expression, as determined by real-time PCR analysis ($\times 1.1$; $P = 0.4293$) (Figure 2A), whereas tumor necrosis factor (TNF)-α expression was significantly reduced ($\times 0.6$, $P < 0.001$) (Figure 2B). IL-10 expression was significantly increased after IL-10 stimulation ($\times 2.5$, $P < 0.001$) (Figure 2C), indicating a positive feedback loop. Furthermore, arginase-1 and CD163, markers for anti-inflammatory macrophages, were significantly up-regulated after IL-10 stimulation (arginase-1: $\times 9.6$, $P < 0.001$; CD163: $\times 4.6$, $P < 0.001$) (Figure 2, D and E). Likewise, we also detected a significant up-regulation of LYVE-1 ($\times 2.0$, $P < 0.01$) (Figure 2F), which is usually expressed by lymphatic vascular endothelial cells and by an incompletely defined macrophage subpopulation implicated in inflammatory hemangiogenesis and lymphangiogenesis.[12,43] In addition, we determined the expression of VEGF-A, a mainly prohemangiogenic growth factor, and VEGF-C and VEGF-D, which predominantly induce the growth of lymphatic vessels. After IL-10 stimulation of PECs, VEGF-A expression decreased ($\times 0.7$, $P < 0.001$) (Figure 2G). Interestingly, expression of the major prolymphangiogenic growth factor VEGF-C was significantly up-regulated by IL-10 ($\times 1.6$, $P < 0.001$) (Figure 2H). VEGF-D expression in PECs was low and remained unchanged after IL-10 stimulation ($\times 1.0$, $P = 0.9805$) (Figure 2I). Thus, IL-10 leads to an anti-inflammatory, antihemangiogenic, but surprisingly prolymphangiogenic phenotype of macrophages.

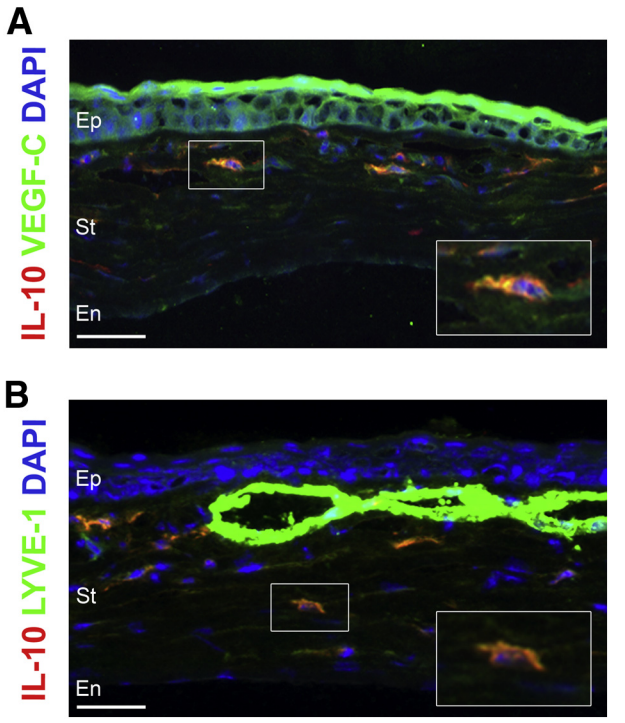

Figure 3 IL-10$^+$ corneal macrophages coexpress vascular endothelial growth factor (VEGF)-C (**A**) and lymphatic vessel endothelial hyaluronan receptor (LYVE)-1 (**B**). Cryosections of inflamed corneas (2 weeks after suture placement) show colocalization of IL-10 with VEGF-C and LYVE-1. **A:** IL-10 (red), VEGF-C (green); additional VEGF-C positivity is detectable in the corneal epithelium. **B:** IL-10 (red), LYVE-1 (green); additional LYVE-1 positivity is detectable in corneal lymphatic vessels. Blue indicates DAPI nuclear staining. Scale bar = 30 μm (**A** and **B**). En, corneal endothelium; Ep, corneal epithelium; St, corneal stroma.

IL-10$^+$ Corneal Macrophages Coexpress VEGF-C and LYVE-1

We next analyzed whether IL-10$^+$ macrophages also express prolymphangiogenic factors in the cornea. Immunohistochemical stainings on inflamed corneas 2 weeks after suture placement showed that IL-10 expression colocalized with VEGF-C and LYVE-1 expression (Figure 3, A and B). Thus, IL-10$^+$ corneal macrophages might be able to contribute to corneal lymphangiogenesis via expression of these factors.

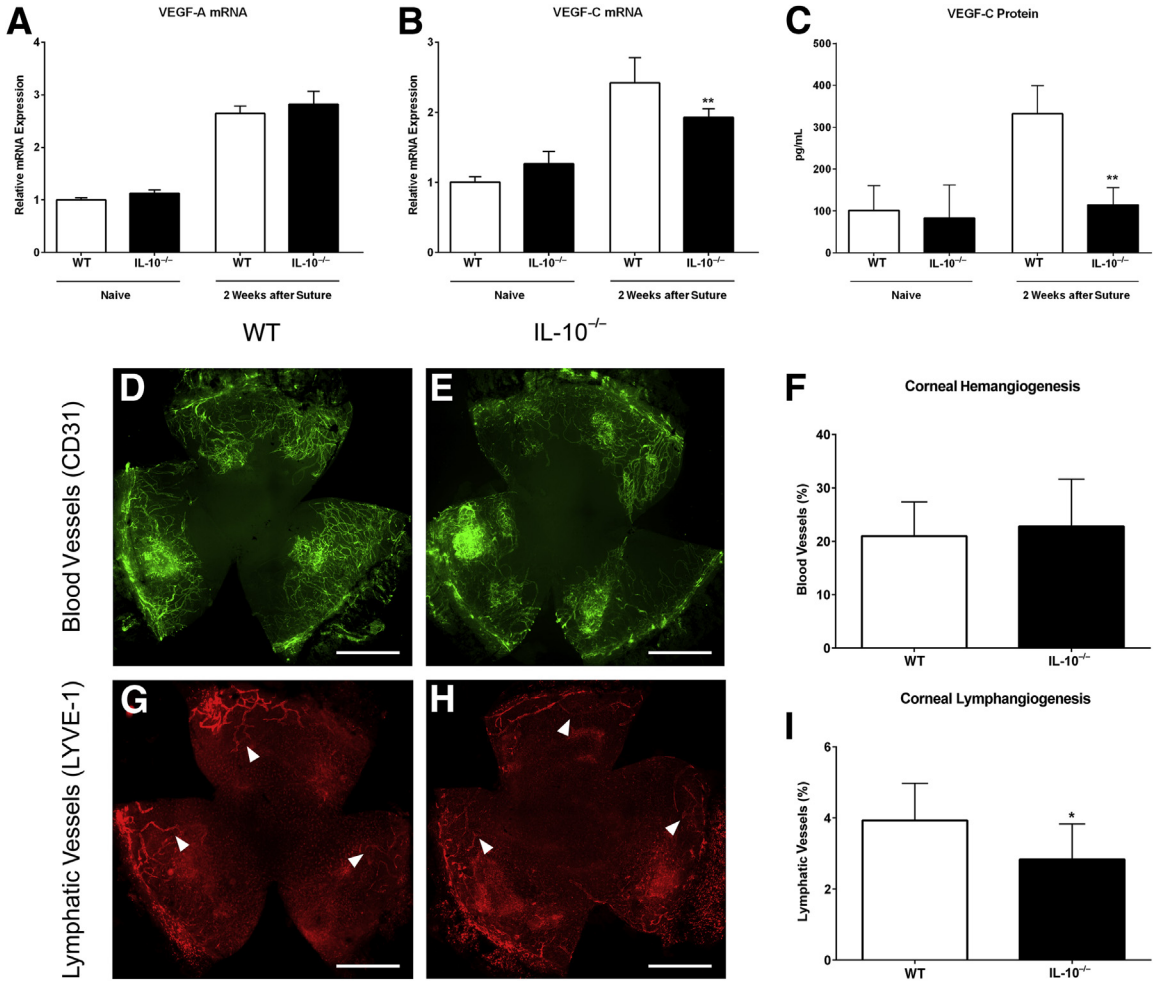

Figure 4 Inflammatory corneal hemangiogenesis and lymphangiogenesis in IL-10–deficient mice. **A** and **B:** Vascular endothelial growth factor (VEGF)-A and VEGF-C mRNA expression in naive and inflamed corneas (2 weeks after suture placement) was assessed in wild-type (WT) and in IL-10–deficient mice (IL-$10^{-/-}$). After suture placement, IL-$10^{-/-}$ shows an increase in corneal VEGF-A mRNA levels that is comparable to WT, whereas the increase in corneal VEGF-C mRNA levels is significantly lower in IL-$10^{-/-}$ compared with WT. **C:** VEGF-C protein levels are significantly reduced in IL-$10^{-/-}$ compared with WT after suture placement. **D–I:** Two weeks after suture placement, corneal hemangiogenesis (**D–F**) is not altered, whereas corneal lymphangiogenesis (**G–I**) is significantly reduced in IL-$10^{-/-}$ compared with WT [corneal whole mounts; blood vessels stained with CD31 (green) and lymphatic vessels stained with LYVE-1 (red)]; **Arrowheads** in **G** and **H** indicate corneal lymphatic vessels. *$P < 0.05$, **$P < 0.01$. Scale bar = 1 mm (**D**, **E**, **G**, and **H**).

IL-10–Deficient Mice Show Less Prolymphangiogenic Growth Factor Expression and Reduced Corneal Lymphangiogenesis after an Inflammatory Stimulus

We next sought to test the prolymphangiogenic effect of IL-10 *in vivo* and therefore performed corneal suture placement in mice deficient in IL-10 (IL-$10^{-/-}$). Naive corneas of IL-$10^{-/-}$ mice showed similar mRNA expression levels of VEGF-A and VEGF-C when compared with WT littermates (VEGF-A: ×1.1; VEGF-C: ×1.2) (Figure 4, A and B). After suture placement, IL-$10^{-/-}$ showed an increase in corneal VEGF-A levels that was comparable to WT (IL-$10^{-/-}$: ×2.8 versus WT: ×2.7) (Figure 4A). However, the increase of corneal VEGF-C mRNA expression after suture placement was significantly lower in IL-$10^{-/-}$ mice when compared with WT (IL-$10^{-/-}$: ×1.9 versus WT: ×2.4; $P < 0.01$) (Figure 4B). Consistently, the increase of corneal VEGF-C protein expression after suture placement was also reduced in IL-$10^{-/-}$ when compared with WT (IL-$10^{-/-}$: mean m = 114.1 pg/mL versus WT: mean = 332.1 pg/mL; $P < 0.01$) (Figure 4C). In addition, morphometric analysis of corneal whole mounts after suture placement revealed that hemangiogenesis was not altered in IL-$10^{-/-}$ mice (mean = +8.7%, SD = 34.3%) (Figure 4, D–F), whereas lymphangiogenesis was significantly reduced in inflamed corneas of IL-$10^{-/-}$ mice (mean = −28.0%, SD = 24.2%; $P < 0.05$) (Figure 4, G–I). Therefore, loss of IL-10 results in less corneal VEGF-C expression, leading to reduced corneal lymphangiogenesis after an inflammatory stimulus.

IL-10 Does Not Directly Affect Proliferation of LECs

Our results point to an indirect regulation of inflammatory corneal lymphangiogenesis by IL-10 via macrophages. To analyze whether IL-10 also directly regulates LECs, we

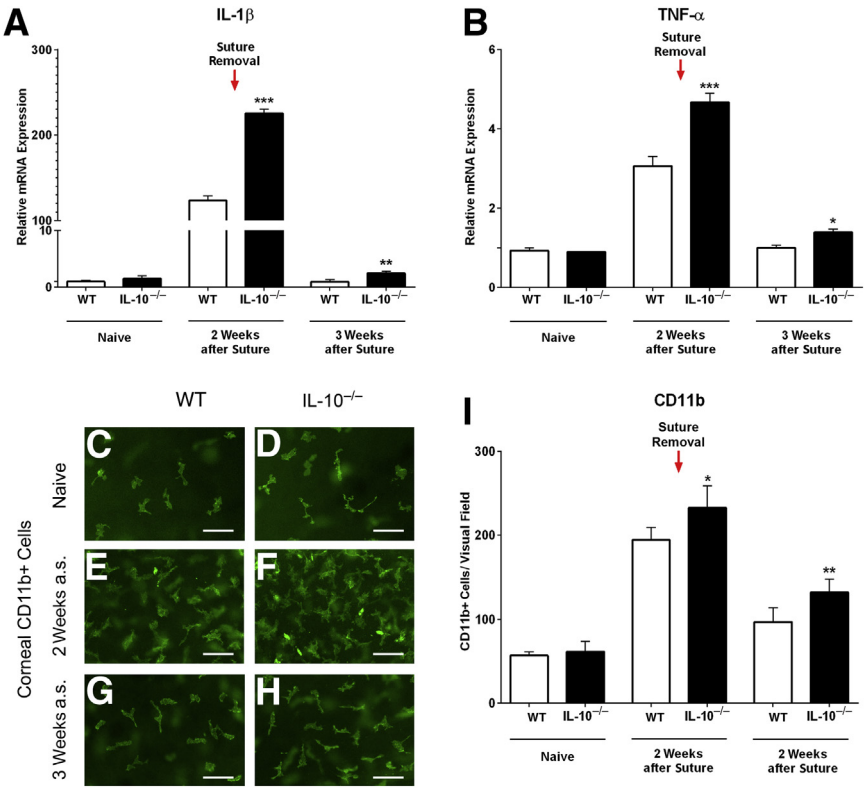

Figure 5 Persistent corneal inflammation in IL-10—deficient mice. **A** and **B:** IL-1β (**A**) and tumor necrosis factor (TNF)-α (**B**) mRNA expression in naive and inflamed corneas (2 and 3 weeks after suture placement) was assessed in wild-type (WT) and IL-10 deficient mice (IL-10$^{-/-}$). After suture placement, IL-1β and TNF-α expression increases to significantly higher levels in IL-10$^{-/-}$ compared with WT. Furthermore, after suture removal (2 weeks after placement), IL-1β and TNF-α expression levels persist on significantly higher levels. **C** and **D:** Corneal CD11b^{+} cell numbers in naïve IL-10$^{-/-}$ (**D**) and WT (**C**) are comparable. **E** and **F:** Two weeks after suture (a.s.) placement, corneal CD11b^{+} cell numbers increase to a significantly higher extent in IL-10$^{-/-}$ (**F**) compared with WT (**E**). **G** and **H:** One week after suture removal (2 weeks after placement), corneal CD11b numbers persist on significantly higher levels in IL-10$^{-/-}$ (**H**) compared with WT (**G**). **I:** Quantification of CD11b numbers during corneal inflammation. **Arrows** in **A**, **B**, and **I:** Suture removal was performed after 2 weeks. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. Scale bar = 20 μm (**C**—**H**).

incubated LECs with VEGF-C, IL-10, or a combination of both, and measured cell proliferation by ELISA. VEGF-C significantly increased LEC proliferation (mean = 19.7%, SD = 18.2%; $P < 0.01$), whereas IL-10 showed no effect (mean = 2.2%, SD = 8.4%). Furthermore, IL-10 did not affect the VEGF-C—mediated increase in LEC proliferation (mean = 16.8%, SD = 14.6%; $P < 0.01$) (Supplemental Figure S1A). In addition, we also determined whether IL-10 regulates VEGF-C and LYVE-1 expression in LECs. Incubation of LECs with IL-10 did not result in significant VEGF-C and LYVE-1 mRNA expression changes, as determined by real-time PCR (VEGF-C: ×1.0; LYVE-1: ×1.2) (Supplemental Figure S1, B and C). Incubation of LECs with VEGF-C also had no significant effect on VEGF-C and LYVE-1 expression (VEGF-C: ×0.9; LYVE-1: ×1.1) (Supplemental Figure S1, B and C). Therefore, we concluded that IL-10 has no direct effect on the proliferation of LECs and does not directly affect VEGF-C and LYVE-1 expression in LECs.

IL-10 Deficiency Leads to Persistent Corneal Inflammation *in Vivo*

Studies in several extraocular tissues, such as the skin, the intestine, and cartilage, have shown that lymphatic vessels play an important role in the resolution of inflammatory responses, and that inflammation persists in absence of lymphatic vessels.[23,44,45] We therefore hypothesized that modulation of lymphangiogenesis by IL-10 might also influence the inflammatory state of the cornea, because fewer lymphatic vessels might lead to retention of inflammatory cells within the cornea. Therefore, we compared the expression of proinflammatory cytokines and the numbers of inflammatory cells of IL-10$^{-/-}$ and WT corneas. Naive corneas of IL-10$^{-/-}$ mice showed similar expression levels of IL-1β and TNF-α when compared with WT littermates (IL-1β: ×1.4; TNF-α: ×0.9) (Figure 5, A and B). However, after suture placement, IL-1β and TNF-α expression increased to significantly higher levels in IL-10$^{-/-}$ compared with WT (IL-1β: IL-10$^{-/-}$: ×225.4 versus WT: ×123.4; $P < 0.001$; TNF-α: IL-10$^{-/-}$: ×4.7 versus WT: ×3.0; $P < 0.001$) (Figure 5, A and B). After suture removal, IL-1β and TNF-α expression levels persisted on significantly higher levels in IL-10$^{-/-}$ when compared with WT (IL-1β: IL-10$^{-/-}$: ×2.5 versus WT: ×1.0; $P < 0.01$; TNF-α: IL-10$^{-/-}$: ×1.4 versus WT: ×1.0; $P < 0.05$) (Figure 5, A and B). In addition, the numbers of corneal CD11b^{+} cells in naïve IL10$^{-/-}$ and WT corneas were similar (IL-10$^{-/-}$: mean = 61.5, SD = 11.9 versus WT: mean = 56.8, SD = 4.6, cells per visual field) (Figure 5, C and D). However, after suture placement, the numbers of corneal CD11b^{+} cells increased to a significantly higher extent in IL-10$^{-/-}$ compared with WT (IL-10$^{-/-}$: mean = 232.8, SD = 26.3 versus WT: mean = 194.2, SD = 15.1, cells per visual field; $P < 0.05$) (Figure 5, E, F, and I). Moreover, 1 week after suture removal, corneal CD11b numbers persisted on significantly higher levels in IL-10$^{-/-}$ compared with WT (IL-10$^{-/-}$: mean = 131.8, SD = 16.0 versus WT: mean = 96.8, SD = 17.0, cells per visual field; $P < 0.01$)

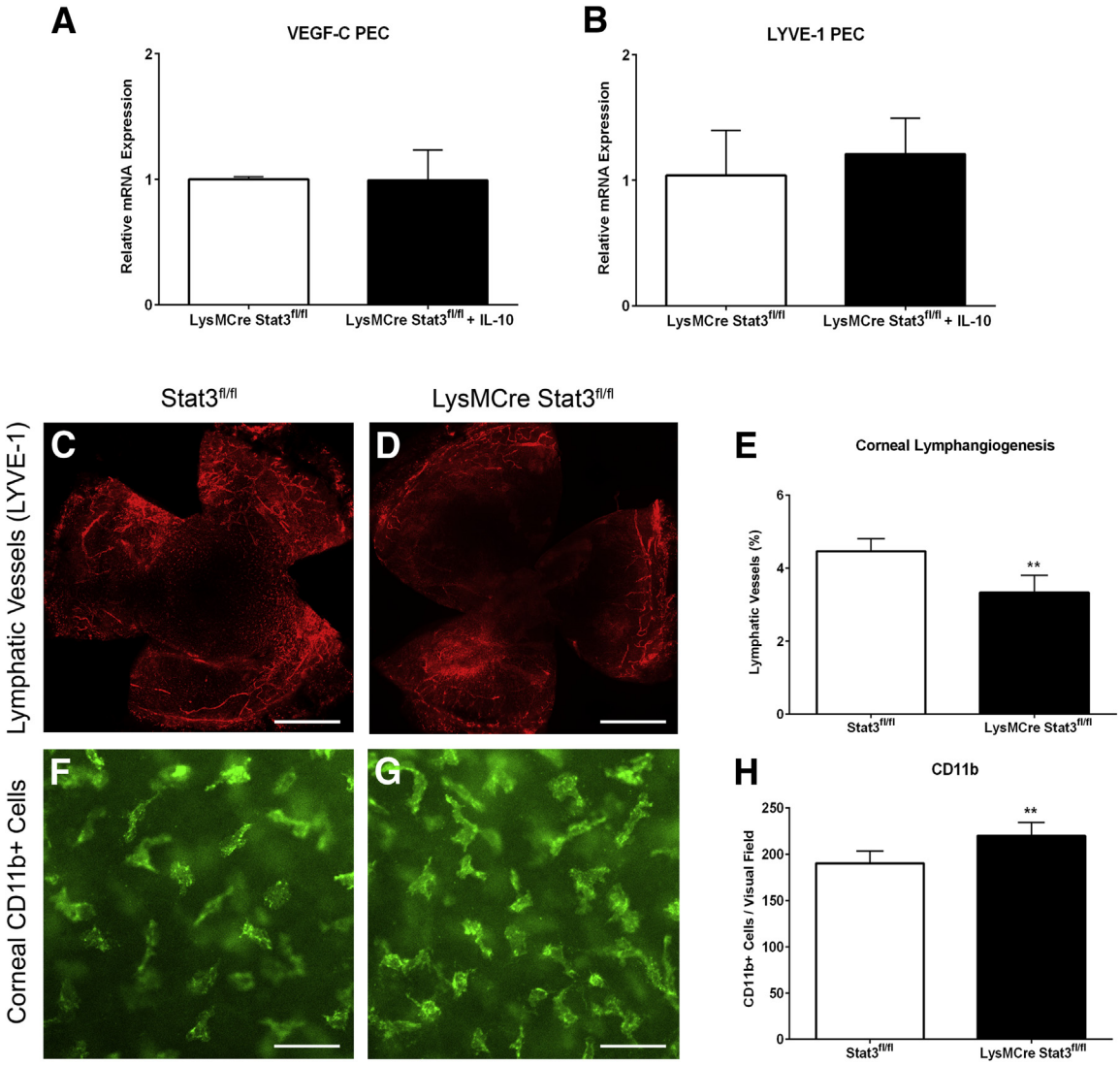

Figure 6 Conditional ablation of Stat3 in myeloid cells leads to decreased corneal lymphangiogenesis and increased corneal inflammation. **A** and **B:** Absent up-regulation of prolymphangiogenic factors in lysozyme M Cre (LysMCre) Stat3$^{fl/fl}$ macrophages. Thioglycollate-activated peritoneal macrophages isolated from LysMCre Stat3$^{fl/fl}$ mice were stimulated with 100 ng/mL IL-10. After 24 hours, mRNA expression levels of vascular endothelial growth factor (VEGF)-C (**A**) and lymphatic vessel endothelial hyaluronan receptor (LYVE)-1 (**B**) remain unchanged when compared with unstimulated macrophages. **C** and **D:** Two weeks after suture placement, corneal lymphangiogenesis is significantly reduced in LysMCre Stat3$^{fl/fl}$ (**D**) compared with Stat3$^{fl/fl}$ (**C**) (corneal whole mounts; lymphatic vessels stained with LYVE-1). **E:** Quantification of corneal lymphangiogenesis. **F** and **G:** Two weeks after suture placement, corneal CD11b^{+} cell numbers increase to a significantly higher extent in LysMCre Stat3$^{fl/fl}$ (**G**) compared with Stat3$^{fl/fl}$ (**F**). **H:** Quantification of corneal CD11b numbers. $^{**}P < 0.01$. Scale bars: 1 mm (**C** and **D**); 20 μm (**F** and **G**). PEC, peritoneal exudate cell.

(Figure 5, G–I). Thus, IL-10 deficiency leads to a more severe inflammatory response at the cornea, which even persists after the inflammatory stimulus has been removed.

Conditional Deletion of Stat3 in Myeloid Cells Leads to Decreased Corneal Lymphangiogenesis and Increased Corneal Inflammation

It has previously been shown that Stat3 plays an important role in IL-10 signaling.[28,34,46] To analyze whether the prolymphangiogenic effect of IL-10 in macrophages also depends on Stat3, we used macrophages isolated from mice in which Stat3 is conditionally deleted in the myeloid cell lineage (LysMCre Stat3$^{fl/fl}$). IL-10 treatment of PECs isolated from LysMCre Stat3$^{fl/fl}$ did not result in a significant up-regulation of the mRNA expression of VEGF-C and LYVE-1 (VEGF-C: ×1.0, LYVE-1: ×1.2) (Figure 6, A and B), indicating that the stimulatory effect of IL-10 on VEGF-C and LYVE-1 expression depends on Stat3. In addition, we performed corneal suture placement in LysMCre Stat3$^{fl/fl}$ and Stat3$^{fl/fl}$. Importantly, LysMCre Stat3$^{fl/fl}$ showed less corneal lymphangiogenesis when compared with control Stat3$^{fl/fl}$ (mean = −25.4%, SD = 10.6%; $P < 0.01$) (Figure 6, C–E). Furthermore, LysMCre Stat3$^{fl/fl}$ showed higher inflammatory cell numbers when compared with control Stat3$^{fl/fl}$ (LysMCre Stat3$^{fl/fl}$: mean = 220.0, SD = 14.4 versus Stat3$^{fl/fl}$:

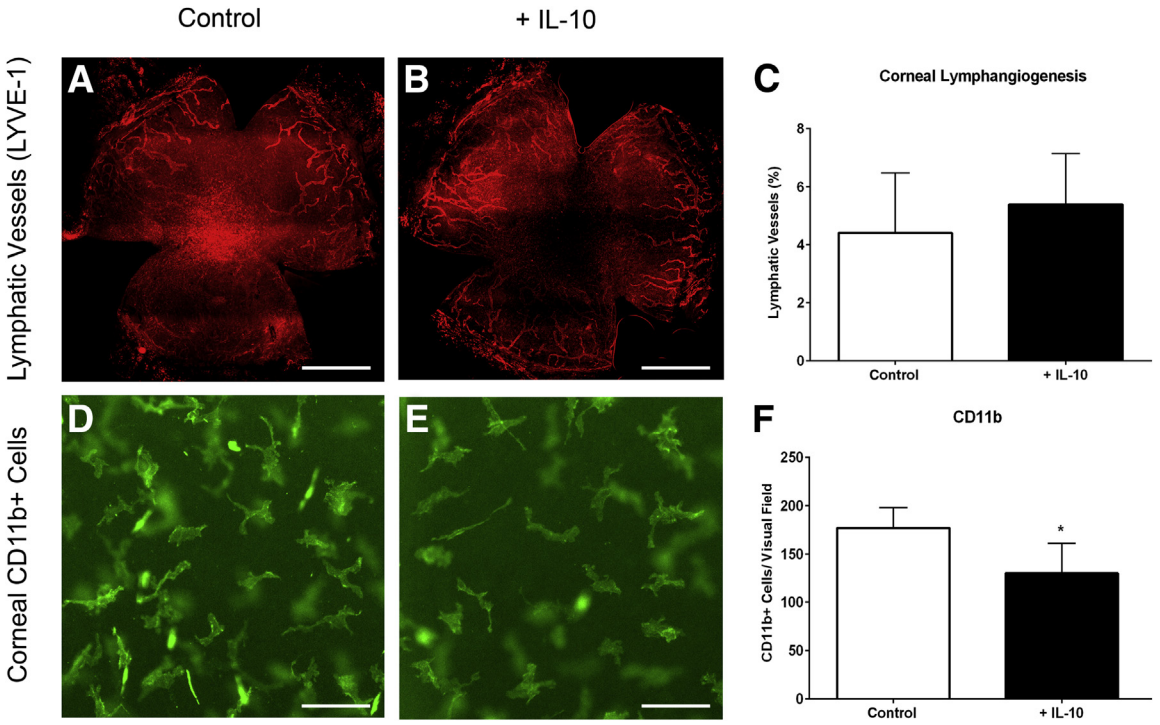

Figure 7 Local treatment with IL-10 promotes lymphangiogenesis and the resolution of corneal inflammation. Two weeks after suture placement in wild-type mice, sutures were removed and mice were treated subconjunctivally with recombinant murine IL-10. **A** and **B:** Corneal lymphangiogenesis is slightly increased in IL-10–treated corneas (**B**) when compared with phosphate-buffered saline (PBS)–treated corneas (**A**), although reaching no significance [corneal whole mounts; lymphatic vessels stained with lymphatic vessel endothelial hyaluronan receptor (LYVE)-1]. **C:** Quantification of corneal lymphangiogenesis after IL-10 treatment. **D** and **E:** After 1 week of treatment, CD11b numbers are significantly lower in IL-10–treated corneas (**E**) when compared with PBS-treated corneas (**D**). **F:** Quantification of CD11b numbers after IL-10 treatment. *$P < 0.05$. Scale bars: 1 mm (**A** and **B**); 20 μm (**D** and **E**).

mean = 190.4, SD = 13.0, cells per visual field; $P < 0.01$) (Figure 6, F–H). Thus, our results show that the prolymphangiogenic polarization of macrophages by IL-10 depends on Stat3. Furthermore, specific deletion of Stat3 in macrophages is sufficient to resemble the corneal phenotype of IL-10$^{-/-}$ mice, confirming the crucial role of IL-10 signaling in macrophages for the regulation of corneal lymphangiogenesis and inflammation.

Local Treatment with IL-10 Promotes Lymphangiogenesis and the Resolution of Corneal Inflammation

Because we had demonstrated that IL-10 regulates corneal lymphangiogenesis and corneal inflammation, we next investigated whether its effects can be used therapeutically to promote the resolution of the inflammatory response at the cornea. For this purpose, we placed corneal sutures in WT mice, which were left in place for 2 weeks until the inflammatory and lymphangiogenic response was reported to reach its maximum.[37,38] Subsequently, sutures were removed and mice were treated with repeated subconjunctival injections of recombinant IL-10. After 1 week of treatment, the amounts of corneal lymphatic vessels and inflammatory cells were determined. Corneal lymphangiogenesis increased in IL-10–treated corneas when compared with PBS-treated corneas, although it reached no significance (mean = 22.2%, SD = 35.6%) (Figure 7, A–C). Moreover, CD11b^{+} cell numbers significantly decreased in IL-10–treated corneas when compared with PBS-treated corneas (IL-10 treated: mean = 130.0, SD = 27.9 versus PBS treated: mean = 177.0, SD = 18.7, cells per visual field; $P < 0.05$) (Figure 7, D–F).

Thus, local treatment with IL-10 seems to modulate lymphangiogenesis and leads to higher egress of inflammatory cells from the cornea, resulting in a faster resolution of corneal inflammation.

Discussion

Herein, we determined the role of IL-10 in inflammatory lymphangiogenesis and the resolution of inflammation using the cornea as a model system. Our results show that IL-10 is not detectable in healthy, but only in inflamed, corneas, and that IL-10 is expressed by infiltrating CD11b^{+} cells, which are mainly corneal macrophages.[12,37,39] It is well known that IL-10 results in an anti-inflammatory macrophage phenotype.[13,29] We therefore analyzed changes in gene expression in IL-10–stimulated macrophages. Consistent with previous studies,[47–49] we found that IL-10 reduced the expression of the proinflammatory cytokine TNF-α and upregulated the expression of arginase-1 and CD163, which are markers for anti-inflammatory macrophages. VEGF-A expression was reduced in IL-10–stimulated macrophages.

Interestingly, expression of the major prolymphangiogenic growth factor VEGF-C was up-regulated in IL-10–treated macrophages. Furthermore, LYVE-1 was also up-regulated, which is particularly interesting because LYVE-1^{+} macrophages seem to be capable to transdifferentiate and integrate into preexisting lymphatic vessels.[12,43,50] In addition, it is possible that expression of LYVE-1, which is a hyaluronan receptor,[51] might facilitate migration of LYVE-1^{+} macrophages through hyaluronan-rich extracellular matrices and might contribute to the generally increased tissue motility of anti-inflammatory macrophages.[52,53] In addition, it was recently demonstrated that LYVE-1 interacts with fibroblast growth factor-2, a potent prolymphangiogenic growth factor.[54,55] The interaction of LYVE-1 with fibroblast growth factor-2 might also affect the migration of macrophages through fibroblast growth factor-2–rich extracellular matrices. Nevertheless, the exact role of LYVE-1 in the regulation of macrophage motility is still undetermined. So far, studies analyzing LYVE-1 function have mainly focused on LYVE-1 expression in LECs and only to a lesser extent on LYVE-1 expression in macrophages. In this context, it is also unknown whether LYVE-1 contributes to tissue homing of macrophages. Nonetheless, LYVE-1–expressing macrophages have recently been detected in a variety of tissues and organs,[43,56–58] and a better understanding of LYVE-1 function in macrophages is expected for the near future.

We demonstrated that inflammatory corneal lymphangiogenesis was reduced in IL-10$^{-/-}$ after corneal suture placement, further supporting our hypothesis that IL-10 has prolymphangiogenic properties *in vivo*. LEC proliferation or LEC gene expression was not directly affected by IL-10, indicating that the effects of IL-10 on lymphangiogenesis are mainly indirect via VEGF-C–secreting, anti-inflammatory macrophages. This is strongly supported by the reduction of corneal lymphangiogenesis in LysMCre Stat3$^{fl/fl}$, where IL-10 does not result in up-regulation of VEGF-C expression in macrophages. IL-10–stimulated prolymphangiogenic macrophages might promote lymphangiogenesis to support their egress from the inflamed site via lymphatic vessels. Consistent with this hypothesis, we observed increased inflammatory cell numbers and more severe inflammation in corneas of IL-10$^{-/-}$ and LysMCre Stat3$^{fl/fl}$, whereas local treatment with IL-10 resulted in decreased corneal inflammatory cell numbers. On the basis of our findings, we propose that during the corneal inflammatory response, IL-10 leads to an anti-inflammatory, prolymphangiogenic polarization of macrophages that induces the activation and growth of lymphatic vessels, which, in turn, support the egress of inflammatory cells and the termination of the local inflammatory response. Because IL-10 stimulation leads to a further up-regulation of IL-10 in macrophages, there seems to be an autocrine amplification cascade incited by IL-10, leading to the termination of inflammation. Nonetheless, our experiments cannot fully rule out that the observed changes in inflammatory cell numbers might also occur independent of lymphatic vessels.

So far, corneal lymphangiogenesis has mainly been investigated in the context of dry eye disease, ocular allergy, and graft rejection after corneal transplantation.[19–21] In these inflammatory diseases, lymphatic vessels grow secondarily into the cornea and seem to enable effective access of antigen-presenting cells to regional lymph nodes, where accelerated antigen sensitization occurs.[5,59] Thus, corneal lymphangiogenesis is mostly considered as harmful, which has led to the development of antilymphangiogenic treatment strategies as a rational and important therapeutic concept in ocular surface diseases.[37,60] In contrast, a physiological, nonpathological function for corneal lymphatic vessels has not been described so far. However, studies in extraocular tissues implicate that lymphatic vessels are important for the termination of physiological inflammatory responses, and that lymphatic vessel activation can ameliorate eg, skin or intestinal inflammation.[24,61] Likewise, our results indicate that lymphatic vessels also have physiological functions at the cornea and also affect the course of corneal inflammation. Therefore, prolymphangiogenic agents might be therapeutically used to stimulate corneal lymphatic vessels and thereby the faster resolution of corneal inflammation. For this purpose, local treatment with IL-10 might be considered as a promising option to promote lymphangiogenesis to terminate inflammatory responses at the cornea.

In summary, we have demonstrated that IL-10 indirectly regulates corneal lymphangiogenesis and the resolution of inflammatory responses via prolymphangiogenic polarized macrophages. This may further support the egress of macrophages from the inflamed tissue via lymphatic vessels and help to terminate an ongoing inflammatory response. IL-10 might be used therapeutically to terminate pathological corneal inflammation.

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Supplemental Data

Supplemental material for this article can be found at *http://dx.doi.org/10.1016/j.ajpath.2015.09.012*.

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