Pathological lymphangiogenesis is modulated by galectin-8-dependent crosstalk between podoplanin and integrin-associated VEGFR-3

Wei-Sheng Chen1,2, Zhiyi Cao2, Satoshi Sugaya2, Maria J. Lopez2, Victor G. Sendra2, Nora Laver2, Hakon Leffler3, Ulf J. Nilsson4, Jianxin Fu5,6, Jianhua Song5,6, Lijun Xia5,6, Pedram Hamrah2 & Noorjahan Panjwani1,2,7

Lymphangiogenesis plays a pivotal role in diverse pathological conditions. Here, we demonstrate that a carbohydrate-binding protein, galectin-8, promotes pathological lymphangiogenesis. Galectin-8 is markedly upregulated in inflamed human and mouse corneas, and galectin-8 inhibitors reduce inflammatory lymphangiogenesis. In the mouse model of corneal allogeneic transplantation, galectin-8-induced lymphangiogenesis is associated with an increased rate of corneal graft rejection. Further, in the murine model of herpes simplex virus keratitis, corneal pathology and lymphangiogenesis are ameliorated in Lgals8−/− mice. Mechanistically, VEGF-C-induced lymphangiogenesis is significantly reduced in the Lgals8−/− and Pdpn−/− mice; likewise, galectin-8-induced lymphangiogenesis is reduced in Pdpn−/− mice. Interestingly, knockdown of VEGFR-3 does not affect galectin-8-mediated lymphatic endothelial cell (LEC) sprouting. Instead, inhibiting integrins α1β1 and α5β1 curtails both galectin-8- and VEGF-C-mediated LEC sprouting. Together, this study uncovers a unique molecular mechanism of lymphangiogenesis in which galectin-8-dependent crosstalk among VEGF-C, podoplanin and integrin pathways plays a key role.
Lymphatic vessels are essential for preservation of fluid balance, nutrient absorption and immune surveillance. Lymphangiogenesis is associated with diverse pathological conditions including metastatic dissemination, solid organ graft rejection, type 2 diabetes, obesity, hypertension, lymphedema and chronic wound healing. Pathological lymphangiogenesis is also associated with various diseases of the eye including corneal graft rejection, herpetic keratitis, dry eye disease, ocular allergy and glaucoma. Indeed, recent studies suggest that lymphatic vessel invasion in and around primary tumours rather than invasion of blood vessels, is the key prognostic marker of the aggressiveness of various types of cancers, and that the growth of lymphatic vessels is also the major reason of corneal graft rejection. The key regulatory signalling axis that induces lymphangiogenesis is vascular endothelial growth factor receptor-3 (VEGFR-3) and its ligand, VEGF-C.

Recent studies have demonstrated that the members of the galectin family of mammalian lectins characterized by a carbohydrate recognition domain (CRD) with affinity for β-galactoside-containing glycans, play a critical role in hemangiogenesis. In this respect, we have shown that a member of galectin family, galectin-3, modulates VEGF-A-induced angiogenic response by binding via its CRD to the N-glycans of integrin αvβ3 and VEGFR-2 and subsequently activating angiogenic signalling pathways. Galectin-8 is a tandem-repeat type member of the galectin family. It contains two different CRDs. The N-terminal CRD prefers α2,3-sialyl glycans and mainly contributes to its unique carbohydrate-binding specificity. In vitro studies have shown that galectin-8 binds to podoplanin (PDPN) and that the lectin promotes adhesion and haptotaxis of lymphatic endothelial cells (LEC)s. However, the direct evidence that galectin-8 exerts its biological functions through PDPN is lacking. In fact, Cueni and Detmar speculated that contribution of the interaction of galectin-8 with PDPN in the modulation of LEC migration and adhesion is most likely minor. Also, based on the findings that both sialylated and extensively glycosylated PDPN-Fc inhibit LEC adhesion and migration in vitro, it has been suggested that the interactions with PDPN ligands on the surface of LECs do not depend on PDPN glycosylation. To date, the biological relevance of carbohydrate-dependent galectin-8/PDPN interactions is still elusive and more direct studies involving the use of galectin-8 knockout (KO) and PDPN KO mice have not been reported.

PDPN is a unique transmembrane receptor protein. It is expressed by LECs but not blood ECs and promotes blood-lymph separation. Mice lacking PDPN have leaky lymphatic vessels and congenital lymphedema. In vitro studies have shown that PDPN expression in LECs is required for lymphatic capillary tube formation as well as VEGF-A-induced cell migration. The critical role of extracellular domain of PDPN in lymphangiogenesis has been demonstrated by studies showing that PDPN-Fc and the functional blocking antibody against extracellular domain of PDPN inhibit LEC migration and tube formation in vitro and suppress lymphangiogenesis in inflamed mouse corneas in vivo. The extracellular domain of PDPN is heavily glycosylated, and O-glycosylation as well as sialylation are critical for PDPN-mediated blood-lymph separation and platelet aggregation.

Integrins are a major class of adhesion receptors. It is well-established that angiogenic signals induced by VEGF and the interplay between VEGF receptors and integrins expressed on endothelial cells play an important role in the process of angiogenesis. Integrins that are well-defined for their role in lymphangiogenesis include αvβ1, αvβ3, α4β1, α5β1 and α9β1 (reviewed in Chen et al.). Activation of integrin β1 by collagen and fibronectin promotes VEGFR-3 activation. Conversely, inhibition of integrin αvβ1 but not αvβ3 attenuates VEGF-C-induced VEGFR-3 activation. In addition to extracellular matrix proteins and growth factor receptors, integrins are glycosylated and interact with distinct members of galectin family in a glycan-dependent manner. Although integrins, VEGFR-3 and PDPN are glycosylated like most cell surface receptors, more direct studies on the role of carbohydrate-dependent function of integrins, PDPN and VEGF-C/VEGFR-3 in the regulation of lymphangiogenesis have not been reported.

In the current study, using multiple approaches involving the use of galectin-8 mutants lacking carbohydrate-binding activity, KO mice, specific sugar inhibitors of galectin-8, and siRNA knockdown of key players of lymphangiogenesis, we establish a critical role of galectin-8 and carbohydrate-mediated recognition in the process of lymphangiogenesis. We demonstrate that galectin-8 expression is markedly upregulated in inflamed corneas, and that galectin-8 is a potent lymphangiogenic factor and a key mediator of VEGF-C signalling. Furthermore, we show that PDPN is a key player in VEGF-C-induced lymphangiogenesis, that knockdown of PDPN interferes with integrin signalling cascades in LECs, and that galectin-8 is a critical mediator of crosstalk among VEGF-C, PDPN and integrin lymphangiogenic pathways. In addition, herpes simplex virus (HSV)-1 infection-induced pathological lymphangiogenesis is reduced in galectin-8 KO mice, and galectin-8-induced lymphangiogenesis is associated with an increased rate of graft rejection in a mouse model of allogeneic corneal transplantation. More importantly, we show here that inhibitors of galectin-8 decrease lymphangiogenesis in inflamed mouse corneas in vivo. This is significant considering that there is much interest in finding ways to inhibit the activities of pro-lymphangiogenic factors for preventing graft rejection, tumour metastasis and other inflammatory conditions.

Results

Galectin-8 is upregulated in inflamed human and mouse corneas. In corneas of patients with graft failure and bacterial keratitis, numerous inflammatory cells were detected in the stroma as highlighted by periodic acid Schiff (PAS) staining (Fig. 1a). Normal corneas expressed little galectin-8 (Fig. 1a). In contrast, robust galectin-8 immunoreactivity was detected in corneas of patients with graft failure and bacterial keratitis. Similarly, in mouse corneas treated with thermal cautery or AgNO3 cautery, intense galectin-8 immunoreactivity was detected in the stromal matrix, whereas in untreated control mouse corneas, galectin-8 expression was minimal (Fig. 1b). In some areas, particularly in the anterior stroma, galectin-8 and type I collagen immunoreactivity colocalized (Fig. 1c). While strong galectin-8 reactivity was detected in lymphatic vessels (CD31+), Fig. 1d). Interestingly, some F4/80+ cells exhibited periodic acid Schiff (PAS) staining, indicating that there is much interest in finding ways to inhibit the activities of pro-lymphangiogenic factors

In inflamed mouse corneas, galectin-8 immunoreactivity was detected in macrophages (F4/80+CD11b+, Fig. 1e) and CD4+ T cells (CD4+CD45+, Fig. 1f). Interestingly, some F4/80+ cells in the posterior corneal stroma were galectin-8+ (Fig. 1e), suggesting that either a subset of F4/80+ cells express galectin-8, or the cells need to be activated to express galectin-8. While it is reasonable to suggest that cells stained positively may be the possible source of the lectin, we note that paracrine actions of galectins have been reported. In this respect, galectins secreted by one cell type may bind to the glycan receptors on the adjacent cells. Therefore, the cells that exhibit immunoreactivity with galectin-8 may not necessarily be the cells that produce the lectin. Taken together, this study demonstrates that galectin-8 is upregulated in inflamed human and mouse corneas.
Galectin-8 promotes lymphangiogenesis \textit{in vivo}. The normally avascular cornea has been extensively used as the \textit{in vivo} model to investigate the molecular mechanism of hemangiogenesis and to examine the efficacy of the inhibitors and activators of hemangiogenesis. In recent years, cornea has also proven to be an invaluable model for defining general mechanisms of lymphangiogenesis. To determine whether galectin-8 promotes lymphangiogenesis, we used the mouse corneal micropocket assay. The vessel area, representing the extent of lymphangiogenesis, was calculated 1 week after galectin-8 pellets were implanted in mouse corneas. The extent of galectin-8-mediated lymphangiogenesis increased in a dose-dependent manner, whereas control pellets had no effect (Fig. 2a, b). To further demonstrate the pro-lymphangiogenic capacity of galectin-8 \textit{in vivo}, we employed another well-established method, the Matrigel plug assay. Matrigels containing galectin-8 or VEGF-C were injected in mice subcutaneously, where they solidified to form plugs. On day 7 post injection, the Matrigel plugs were harvested and frozen sections of the plugs were stained with anti-LYVE-1 to visualize lymphatic vessels. As expected, VEGF-C stimulated the growth of new lymphatic vessels in Matrigel, whereas PBS (control) did not (Supplementary Fig. 1a and b).
Supplementary Methods). Similar to VEGF-C, galectin-8 also promoted robust lymphangiogenesis in Matrigel (Supplementary Fig. 1a). To determine the mitogenic effect of galectin-8 on LECs in vivo, corneal micropocket assays were performed using VEGF-C, galectin-8 or control pellets in Prox1-EGFP (enhanced green fluorescent protein) reporter mice. On day 7 post surgery,
corneas implanted with various pellets were stained with anti-Ki67. In control corneas, <4% of LECs (Prox1+ cells) were Ki67+. In contrast, more than 35% of LECs were Ki67+ in both VEGF-C- and galectin-8-induced lymphangiogenic areas (Supplementary Fig. 1b and Supplementary Methods). These data lead us to conclude that galectin-8 is a pro-lymphangiogenic factor as assessed by two independent in vitro methods.

**Galectin-8 promotes LEC sprouting in vitro.** To characterize the role of galectin-8 in the regulation of phenotypic behaviour of LECs in vitro, we examined the effect of galectin-8 on LEC proliferation, migration, tube formation and sprouting. In contrast to in vivo results, galectin-8 treatment had no effect on LEC proliferation in vitro (Supplementary Fig. 1c and Supplementary Methods). We reason that continuously produced galectin-8 may be required to stimulate LEC proliferation in vitro, as has been observed for other (lymph)angiogenic factors such as angiopoietin-1 (refs 28,29) and sphingosine-1-phosphate30. Galectin-8, however, promoted cell migration and tube formation (Supplementary Fig. 1d,e and Supplementary Methods). To better characterize the molecular mechanism by which galectin-8 mediates lymphangiogenesis, we utilized an in vitro three-dimensional LEC sprouting assay. In the sprouting assay, galectin-8, but not galectins-1, 3 or 7, promoted LEC sprouting (Fig. 2c). The stimulatory effect of galectin-8 on LEC sprouting was concentration-dependent (Fig. 2d,e). Next, we tested whether the stimulatory effect of galectin-8 on LEC sprouting was carbohydrate-dependent. First, galectin-8-induced LEC sprouting was almost completely inhibited by thiodigalactoside (TDG), a pan inhibitor of galectins, whereas sucrose, a non-inhibiting disaccharide for galectins, had no effect (Fig. 2d). The pH (Hill coefficient) of galectin-8-induced LEC sprouting was 3.7, indicating a positively cooperative effect of galectin-8-induced LEC sprouting (Fig. 2e). Secondly, compared with wild-type (WT) galectin-8, a galectin-8 mutant, Gal-8Q47A, which has markedly reduced ability to bind 2,3-sialylated glycans13,31, required at least 10 times higher concentration to promote LEC sprouting (Fig. 2f). Thirdly, 3-sialyllactose (3’-SL, 10 mM), which binds N-CRD but not C-CRD of galectin-8 (ref. 13), markedly inhibited galectin-8-induced LEC sprouting, whereas 6’-SL, which does not bind galectin-8, had no effect (Fig. 2g and Supplementary Fig. 2a–c). These data establish that the stimulatory effect of galectin-8 on LEC sprouting is carbohydrate-dependent and that N-CRD of galectin-8 plays a critical role in the process of galectin-8-induced lymphangiogenesis.

Next, we tested whether N-CRD can serve as a dominant negative inhibitor of galectin-8. Di/multivalent property of galectins allow them to crosslink many cell surface and extracellular matrix glycoproteins, such as integrins and growth factor receptors, to regulate signal transduction pathways37. Isolated CRDs, which retain their carbohydrate-binding ability but are unable to dimerize or oligomerize and crosslink cell surface receptors, may compete with the carbohydrate-binding ability of the endogenous galectins and, hence, act as a dominant negative inhibitor16.33. Published studies have shown that isolated CRDs of galectin-8 retain the carbohydrate-binding activity but manifest impaired biological activity13,34, suggesting that the biological function of the lectin is dependent on cooperative interactions of the two CRDs. As described before, N-CRD of galectin-8 (Gal-8N) is unique among galectins in exhibiting a very high affinity for 2,3-sialyl glycans11–13. To determine whether the pro-lymphangiogenic property of galectin-8 is dependent on the cooperative action of both CRDs, we tested whether N-CRD is able to promote LEC sprouting. Unlike full-length galectin-8, Gal-8N failed to induce LEC sprouting (Fig. 2h). Moreover, Gal-8N effectively inhibited galectin-8-induced LEC sprouting (Fig. 2i and Supplementary Fig. 2d–f). These results suggest that Gal-8N serves as a dominant negative inhibitor of galectin-8 and that 2,3-sialyl glycans recognized by Gal-8N as well as cooperative action of both CRDs are required for galectin-8-induced LEC sprouting.

**AKT and ERK1/2 are essential to lymphangiogenesis**35,36. We, therefore, tested the activation of AKT and ERK1/2 pathways by galectin-8. Galectin-8 induced phosphorylation of AKT and ERK1/2 in a time- and dose-dependent manner (Supplementary Fig. 3).

**Galectin-8 modulates pathological lymphangiogenesis in vivo.** Since lymphangiogenesis contributes to corneal graft rejection2,37,38, we sought to determine if galectin-8-induced lymphangiogenesis promotes graft rejection in a mouse model of corneal transplantation. To test this, we performed allogeneic corneal transplantation (donor, male C57BL/6 mice; recipient, male BALB/c mice; both 10 weeks old) and treated the recipient mice with recombinant galectin-8 or PBS (subconjunctivally and intraperitoneally, twice a week beginning at day 7). On postoperative week 4, graft survival rate was markedly decreased in galectin-8-treated mice compared with PBS-treated mice (survival rates: PBS, 66.7%, 14 out of 21; galectin-8, 18.5%, 5 out of 27) (Fig. 3a). Excised corneas from each group were assessed for the extent of corneal lymphangiogenesis on postoperative week 4. The extent of lymphangiogenesis in galectin-8-treated corneas was significantly higher than that of control corneas (Fig. 3b). These results support the notion that galectin-8-induced lymphangiogenesis promotes graft rejection.

Next, to determine the role of endogenous galectin-8 in lymphangiogenesis in vivo, we utilized two different mouse models of pathological lymphangiogenesis in WT and galectin-8 KO mice. HSV-1 keratitis is one of the most common ocular infections. It is characterized by recurrent episodes and is the leading cause of infectious corneal blindness in the developed countries39. HSV-1 infection drives corneal lymphangiogenesis20 and inhibiting lymphangiogenesis is thought to be of potential therapeutic value to alleviate corneal pathology caused by HSV-1 infection40. To determine if endogenous galectin-8 plays a role in HSV-induced pathology and lymphangiogenesis, we infected mouse corneas of WT and galectin-8 KO mice with a clinical isolate of HSV-1. As expected, on day 8 post infection, HSV-1 infection caused corneal opacification (Fig. 3c) and induced corneal lymphangiogenesis (Fig. 3d). In contrast, galectin-8 deficiency not only ameliorated corneal opacity (Fig. 3c) but also reduced corneal lymphangiogenesis (Fig. 3d), suggesting that galectin-8 is involved in the pathogenesis of HSV keratitis, at least partly, by regulating pathological lymphangiogenesis.

**Suture placement in the mouse cornea is a commonly used technique to determine inflammatory lymphangiogenesis in the setting of sterile condition** (in contrast to infectious agent-induced lymphangiogenesis)41,42. Clinical relevance of the method stems from the fact that suture placement is routinely used in many corneal surgeries. In this model also, the extent of suture-induced lymphangiogenesis was reduced in the galectin-8 KO mice (Fig. 3e). Together, these findings conclusively demonstrate that galectin-8 is required for robust pathological lymphangiogenesis.

**Effect of galectin-8 on VEGF-C-induced LEC sprouting and lymphangiogenesis.** To gain mechanistic insight on galectin-8-mediated LEC sprouting and lymphangiogenesis, first we tested whether the lectin influences the function of a well-known
lymphangiogenic molecule, VEGF-C. VEGF-C-induced LEC sprouting was inhibited by galectin-8 inhibitors: TDG (a pan inhibitor of galectins, Fig. 4a,b), 3′-sialyl lactose (the high affinity ligand of the N-CRD of galectin-8, Fig. 4c,d), and by Gal-8N (the dominant negative inhibitor of galectin-8; Fig. 4e,f). These data establish the critical role of galectin-8-dependent carbohydrate-mediated recognition in VEGF-C-induced LEC sprouting.

**Figure 3 | Galectin-8 modulates ocular lymphangiogenesis and associated pathology.** (a,b) Galectin-8 promotes corneal graft rejection. (a) BALB/c mice (N = 48) were transplanted with corneal allografts from C57BL/6 mice. Animals were treated with galectin-8 (10 μg for subconjunctival injection and 50 μg for intraperitoneal injection, N = 27) or PBS (N = 21) twice a week beginning postoperative week 1. Grafts were evaluated weekly for signs of rejection by slit-lamp biomicroscopy. Representative photomicrographs of accepted or rejected allografts are shown in the right panel. Kaplan–Meier survival curve demonstrates that galectin-8 promotes allograft rejection. (b) On postoperative week 4, PBS-treated corneas (N = 13) or galectin-8-treated corneas (N = 14) were harvested and corneal flat mounts were stained with anti-LYVE-1, and lymphatic vessel areas were quantified (left). Representative fluorescence images are shown in the right panel. Data are plotted as mean ± s.e.m. and analysed using Log-rank (Mantel–Cox) test (a) and Student’s t-test (b). **P < 0.01 versus PBS. (c,d) Lymphangiogenesis and severity of HSV keratitis is reduced in galectin-8 KO mice. WT (N = 13) and galectin-8 KO (N = 8) mouse corneas were scarified and infected with 1 × 10⁶ pfu of HSV-1. The progression of HSV keratitis was assessed by corneal opacity scores on day 3, 6 and 8 post infection (c). Representative photomicrographs of eyes on day 8 post infection are shown in the right panel. (c) Suture-induced inflammatory lymphangiogenesis is reduced in galectin-8 KO mice. Sutures were placed 2 mm above the limbal vessel in the corneas of WT (N = 9) and galectin-8 KO (N = 9) mice. After 7 day post-surgery, the corneal flat mounts were stained with anti-LYVE-1 and lymphatic vessel areas were quantified. Representative fluorescence images are shown in the right panel. Data are plotted as mean ± s.e.m. and analysed using Student’s t-test. *P < 0.05, **P < 0.01, ***P < 0.001 versus PBS (b) or WT (c,d,e). Scale bars: 800 μm (b,d); 200 μm (e).
are expressed as a change with respect to VEGF-C-treated LEC spheroids. Representative images are shown in the right panel. (b) Synergistic effect of galectin-8 on VEGF-C-induced LEC sprouting is dose-dependent. The values for inhibitor-treated groups are expressed as a change with respect to VEGF-C-treated LEC spheroids. Representative fluorescent images are shown in the right panel. Black asterisk, control pellet; red asterisk, VEGF-C pellet; blue asterisk, galectin-8 pellet. (c) VEGF-C-induced lymphangiogenesis is reduced in galectin-8 KO mice. VEGF-C pellets (160 ng) were implanted into WT (N = 11) and galectin-8 KO (N = 11) mice. After 7 day post implantation, the corneal flat mounts were stained with anti-LYVE-1 and lymphatic vessel areas were quantified. Asterisks indicate VEGF-C pellets. Data are plotted as mean ± s.e.m. and analysed using one-way ANOVA (g,h) and Student’s t-test (a,c,e). ***P < 0.001 versus control (g), VEGF-C (a,c,e). #P < 0.05, ##P < 0.01, ###P < 0.001 versus VEGF-C (g). ANOVA, analysis of variance; TDG: thiodigalactoside; 3′-SL, 3′-sialyllactose; Gal-8N, N-terminal CRD. The results are representative of three or more independent experiments. Scale bars: 100 μm (b,d,f,h); 400 μm (i); and 200 μm (j).
To further determine the effect of galectin-8 on VEGF-C-induced LEC sprouting, we tested the effect of exogenous galectin-8 on VEGF-C-induced LEC sprouting. Galectin-8, but not galectins-1, 3 and 7, markedly enhanced VEGF-C-induced LEC sprouting (Fig. 4g). In addition, at 0.75 μM of galectin-8, VEGF-C-induced LEC sprouting was five times higher than that seen by VEGF-C alone or galectin-8 alone (Fig. 4h), indicating that galectin-8 has a synergistic effect on VEGF-C-induced LEC sprouting. In the in vivo micropocket assays also, galectin-8 collaboratively augmented VEGF-C-induced lymphangiogenesis (Fig. 4i). Moreover, in galectin-8 KO mice, the extent of VEGF-C-induced lymphangiogenesis was significantly reduced (Fig. 4j).

Galectin-8- and VEGF-C-induced LEC sprouting is dependent on PDPN. It has been reported that cell surface receptor clustering by the galectin-glycan lattices increases the magnitude or duration of signalling from the cell surface33,44. Therefore, in an attempt to characterize the mechanism by which galectin-8 modulates VEGF-C-induced lymphangiogenesis, we first conducted a study to determine whether the lectin modulates VEGFR-3, the predominant VEGF-C receptor. This study revealed that VEGFR-3, but not VEGF-C, is a galectin-8-binding protein (Supplementary Fig. 4a,b), and that galectin-8 clusters VEGFR-3 on cell surface (Supplementary Fig. 4c). However, surprisingly, knockdown of VEGFR-3 had little effect on galectin-8-induced LEC sprouting (Supplementary Fig. 5a–c), suggesting that molecules besides VEGFR-3 are involved in galectin-8-induced LEC sprouting. Therefore, we sought to determine whether galectin-8-mediated LEC sprouting involves other receptors for VEGF-C. In this respect, it is known that VEGFR-2, which also binds VEGF-C, is not involved in VEGF-C-induced sprouting45,46. Our siRNA knockdown and/or antibody blocking studies revealed that several other known receptors of VEGF-C including neuropilin-2 and integrin αvβ1 (refs 47,48) are not possible targets of galectin-8 (Supplementary Figs 5d–f and 6).

Next we performed studies to determine whether galectin-8-induced LEC sprouting is dependent on PDPN. PDPN is thought to play a role in lymphangiogenesis19,20. However, the role of galectin-8 in the modulation of PDPN has thus far not been fully investigated and virtually nothing is known about the role of PDPN in VEGF-C-induced lymphangiogenesis. Here, we first showed that PDPN expressed in LECs interacts with galectin-8, but not galectins-1, 3 or 7 (Fig. 5a), and the binding of PDPN to galectin-8 was carbohydrate-dependent (Fig. 5b). In addition, PDPN expressed in LECs contains α2,3-sialylated glycans (Fig. 5b). Removal of α2,3-sialylated glycans by treatment with α2-3 neuraminidase abrogated the interaction of PDPN and galectin-8, suggesting that galectin-8 binds α2,3-sialylated glycans of PDPN (Supplementary Fig. 6 and Supplementary Methods).

Secondly, to determine whether PDPN indirectly regulates the functions of VEGF-C/VEGFR-3 through controlling the function of integrins αvβ1 and αvβ5 in galectin-8-dependent manner, we performed studies to determine whether: (i) PDPN inhibition attenuates matrix-mediated LEC migration; (ii) PDPN interacts with integrins αvβ1 in a galectin-8-dependent manner; and (iii) knockdown PDPN impedes integrin-mediated signalling cascades. In this study, blocking the function of PDPN by antibodies as well as siRNA knockdown attenuated both fibronectin- and galectin-8- promoted cell migration (Fig. 6b). These data in conjunction with a published study15 showing that PDPN-Fc inhibits type I collagen-mediated LEC migration, suggest that PDPN is involved in not only galectin-8 but also fibronectin- and type I collagen-mediated LEC migration, a process in which integrins are well-known to play a key role. To assess the galectin-8-dependent interaction between integrin β1 and PDPN, primary LECs were treated with galectin-8 for 15 min, fixed without permeabilization, stained with antibodies against integrin β1, PDPN and galectin-8, and examined by confocal microscopy. In untreated control cells, galectin-8, integrin β1 and PDPN were homogenously distributed all over the LECs (Fig. 6c). Since we showed that galectin-8 was upregulated in inflamed corneas (Fig. 1), we added the exogenous galectin-8 to see whether galectin-8 changes the distribution of PDPN and/or integrin β1 on LECs. Addition of galectin-8 caused dramatic redistribution and clustering of PDPN and integrin β1 on LEC plasma membrane (Fig. 6d). To more directly assess the association between integrins and PDPN, lysates from untreated or galectin-8-treated LECs were incubated with anti-PDPN antibody, and immunoprecipitated proteins were examined by western blotting using antibodies against specific integrins, galectin-8 and PDPN. In untreated cell lysates, immunoprecipitation with anti-PDPN co-immunoprecipitated endogenous galectin-8 and specific integrins (αv, α5, αv, β1, but not α9 or β3), indicating that PDPN interacted with endogenous galectin-8 and the association between PDPN and specific integrins (αv, α5, αv and β1) was constitutive (Fig. 6e). When cells were treated with exogenous galectin-8, there was an increased association between PDPN and integrins α5, αv, β1, while the association between PDPN and integrin αv remained similar (Fig. 6e).

Next, to assess the role of PDPN on integrin-mediated signalling, LECs transfected with control or PDPN siRNA were
Figure 5 | Galectin-8- and VEGF-C-induced lymphangiogenesis is dependent on PDPN. (a) PDPN binds to galectin-8, but not to galectins-1, 3 or 7. (b) Galectin-8 binds to PDPN in a carbohydrate-dependent manner. LEC lysates were incubated with galectin-8 conjugated to agarose beads in the presence or absence of lactose or sucrose (100 mM) and MAA II conjugated to agarose beads. Bound proteins were examined along with total cell lysates (input) by western blot using anti-PDPN. (c) PDPN knockdown: primary LECs were transfected with control (mock) or two siRNA that targeted different regions of PDPN. Knockdown efficiency was assessed by western blot using anti-PDPN and anti-β-actin antibodies. (d–e) PDPN knockdown inhibits VEGF-C- as well as galectin-8-induced LEC sprouting. Spheroids prepared using primary LECs transfected with control or pooled PDPN siRNA were treated with galectin-8 (0.75 μM) or VEGF-C (50 ng ml⁻¹). After 24 h, accumulated sprout lengths were quantified (d). Representative images of the sprouts are shown in the right panel. (f) PDPN knockdown significantly decreases galectin-8-induced as well as VEGF-C-induced activation of AKT but not ERK. Primary LECs were transfected with control or PDPN siRNA. The cells were serum-starved and treated with galectin-8 (0.5 μM) or VEGF-C (50 ng ml⁻¹, positive control) for 30 min. Electrophoresis blots of cell lysates were probed with indicated antibodies. Quantification of fluorescence intensity of western blots (N = 5) is shown in right panels. (g,h) PDPN deficiency diminishes VEGF-C- and galectin-8-induced lymphangiogenesis. VEGF-C pellets (160 ng) (N = 10) (g) and galectin-8 pellets (160 ng) (N = 9) (h) were implanted into WT and PDPN inducible KO mice. After 7 days post implantation, the corneal flat mounts were stained anti-LYVE-1 and lymphatic vessel areas were quantified as described in Methods. Representative fluorescence images from each group are shown. Asterisks indicate pellets. Data are plotted as mean ± s.e.m. and analysed using Student’s t-test. *P < 0.05, **P < 0.01, ***P < 0.001 versus corresponding control or WT mice. Scale bars: 100 μm (e); and 200 μm (g,h).
Figure 6 | Integrins regulate galectin-8-mediated LEC sprouting and migration. (a) αβ1 and α5β1 inhibition reduces both VEGF-C- and galectin-8-induced LEC sprouting. LEC spheroids were stimulated with VEGF-C or galectin-8 in the presence or absence of control IgG, blocking antibodies and peptides. Obtustatin: specific for integrin αβ1; BIO1211: specific for integrin α4β1. A value of 1.0 was assigned to the sprout length of VEGF-C or galectin-8-treated LEC spheroids. (b) Matrix-mediated LEC migration is dependent on PDPN. LECs incubated with anti-PDPN Ab (left), or transfected with PDPN siRNA (right) were seeded into the upper chamber of Transwell inserts. The lower side of the insert membrane was coated with fibronectin or galectin-8-treated LEC spheroids. (c, d) Galectin-8 clusters integrin αβ1 and PDPN on cell surface. LECs were treated with or without galectin-8 for 15 min, fixed without permeabilization, stained with antibodies to integrin αβ1 (green), PDPN (blue) and galectin-8 (red), and examined by confocal microscopy. (e) Control LECs. Exogenous galectin-8 sequesters integrin αβ1 and PDPN (white spots in the merged image) after galectin-8 treatment. Scale bar, 7.5 μm. (e) Integrins α5 and β1 interact with PDPN in a galectin-8-dependent manner. LECs were incubated with or without galectin-8 (0.5 μM) for 15 min at 37 °C. Cell lysates were immunoprecipitated with control or anti-PDPN antibodies, and were processed for western blotting using antibodies indicated. (f) Cell surface expression of integrins α5, β1 and VEGFR-3 remains similar in PDPN knockdown cells. Flow cytometry analysis was used to assess cell surface expression levels of PDPN, VEGFR-3, integrins αβ1 and α5 in LECs transfected with control siRNA (blue line) or PDPN siRNA (red line). Black lines: cells stained with isotype IgG. Data are plotted as mean ± s.e.m. and analysed using one-way ANOVA (a,b) and Student’s t-test (b). ****P<0.0001 versus VEGF-C or Gal-8 (a); ##P<0.001 versus VEGF-C or Gal-8 + control IgG (a). *P<0.05, **P<0.01 versus control IgG (b); ***P<0.001 versus control siRNA (b); ###P<0.01 versus control IgG (b). ANOVA, analysis of variance.
seeded on collagen I- or fibronectin-coated wells and allowed to adhere for 15 min at 37°C. Cell lysates from attached cells on collagen I or fibronectin were analysed with western blotting using phospho-specific integrin and focal adhesion kinase (FAK) antibodies. Phosphorylation of integrin β1 was reduced in PDPN knockdown cells (Supplementary Fig. 7), suggesting that the activation of integrin β1 is reduced in the absence of PDPN. Also, phosphorylation of FAK was decreased in the PDPN knockdown cells seeded on collagen I- but not fibronectin-coated wells (Supplementary Fig. 7), whereas phosphorylation of ERK was markedly reduced in the PDPN knockdown cells seeded on both matrix proteins (Supplementary Fig. 7). To eliminate the possibility that the loss of integrin activation and signalling in the PDPN knockdown cells was not due to altered expression and cellular distribution of integrins and VEGFR-3, we performed studies to determine whether PDPN knockdown alters the cell surface expression of integrins and/or VEGFR-3, using two different approaches: cell surface biotinylation and flow cytometry analysis. In the cell surface biotinylation approach, GAPDH and Prox1 were not detected in the streptavidin pulldown cell lysates, and cell surface PDPN was markedly reduced in the PDPN knockdown cells (Supplementary Fig. 8 and Supplementary Methods). Expression of cell surface VEGFR-3, integrins α5 and β1 was similar in the PDPN knockdown cells and control cells (Supplementary Fig. 8). Similarly, in the flow cytometry analysis approach, cell surface expression of PDPN was reduced, whereas that of VEGFR-3, integrins α5 and β1 did not change in the PDPN knockdown cells and control cells (Supplementary Fig. 8). Similarly, in the flow cytometry analysis approach, cell surface expression of PDPN was reduced, whereas that of VEGFR-3, integrins α5 and β1 did not change in the PDPN knockdown cells and control cells (Supplementary Fig. 8). Similarly, in the flow cytometry analysis approach, cell surface expression of PDPN was reduced, whereas that of VEGFR-3, integrins α5 and β1 did not change in the PDPN knockdown cells and control cells (Supplementary Fig. 8).

Figure 7 | Galectin inhibitors markedly decrease inflammatory lymphangiogenesis in vivo. (a–f) Sutures were placed ~2 mm above the limbic vessel in the corneas of the Prox1-EGFP reporter mice (N = 12 or more). (g–l) Silver nitrate cautery was introduced in the centre of the corneas of the Prox1-EGFP reporter mice (N = 5 or more). The animals were treated with TDG (200 mM in 10 μL, a pan inhibitor of galectins) (c, i), or Gal-8N (15 μg in 10 μL, a dominant negative inhibitor of galectin-8) (f, l) by local subconjunctival injections on days 0, 2, 4 and 6 post-surgery. At the end of the treatment period, lymphatic vessel areas were quantified (a, d, g, j). Representative images are shown in the bottom panels. Data are plotted as mean ± s.e.m. and analysed using Student’s t-test. The results are representative of two independent experiments. Scale bar: 200 μm (b, c, e, f); and 1 mm (h, i, k, l).
differentially expressed. Taken together, these data lead us to conclude that PDPN regulates the functions of integrin $\alpha_1\beta_1$ complexes, specifically of integrins $\alpha_1\beta_1$ and $\alpha_5\beta_1$ in LECs, and that this function is galectin-8-dependent.

**Galectin-8 inhibitors decrease inflammatory lymphangiogenesis.**

To determine whether galectins can be targeted to control lymphangiogenesis, two in vivo models of lymphangiogenesis were used. After suture placement and AgNO$_3$ cauterization in the corneas of Prox1-EGFP reporter mice to induce inflammation, the mice were treated with TDG (200 mM, a pan inhibitor of galectins) or Gal-8N (15 $\mu$g, the dominant negative inhibitor of galectin-8) by subconjunctival injections on days 0, 2, 4 and 6 post surgery. At the end of the treatment period, lymphatic vessel areas were quantified. Treatment with both TDG (Fig. 7a,c,g,i) as well as Gal-8N (Fig. 7d,f,j,l) significantly suppressed suture- and cauterity-induced corneal lymphangiogenesis. These data suggest a promising new mechanism for the modulation of pathological lymphangiogenesis by targeting galectin-8.

**Discussion**

We demonstrate here that galectin-8 is highly upregulated in pathological corneas and plays a critical role in the process of lymphangiogenesis. The striking finding that several other members of galectin family including galectins-1 and 3, which are known to promote hemangiogenesis, did not promote LEC sprouting suggests that galectin-8-mediated LEC sprouting involves the affinity of N-CRD of galectin-8 for 3'-sialylated galactosides that is unique among animal galectins 11–13. In support of this notion, specific inhibition of the N-CRD of galectin-8 with 3'-SL reduced LEC sprouting and a galectin-8 mutant, Gal-8Q47A, which has lost its ability to bind to $\alpha_2,3$-sialyl glycan, did not promote LEC sprouting. Together, these data establish that galectin-8 promotes LEC sprouting in a carbohydrate-dependent manner and that N-CRD of galectin-8 was directly involved in the stimulatory effect of galectin-8 on LEC sprouting.

A major finding of the current study is that galectin-8 modulates VEGF-C-mediated lymphangiogenesis. Our studies
show that Gal-8N (the dominant-negative inhibitor of galectin-8) and 3′-SL (a competing disaccharide) ameliorated VEGF-C-induced LEC sprouting. Furthermore, in the in vivo corneal micropocket assay, the extent of lymphangiogenesis induced by VEGF-C was significantly less in galactin-8 KO mice compared with the WT mice. To our knowledge, this is the first demonstration of a defect in lymphangiogenic response of galactin-8 KO mice. In addition, exogenous galactin-8 markedly enhanced VEGF-C-induced LEC sprouting in vitro and lymphangiogenesis in vivo in a carbohydrate-dependent manner. Together, these data conclusively establish that galactin-8 significantly influences VEGF-C-mediated lymphangiogenesis.

Of note, the inhibitory effect of Gal-8N on VEGF-C-induced LEC sprouting is bell-shaped, which is similar to several other anti-(lymph)angiogenic molecules such as RGDF-mimetic integrin inhibitors (ref. 50). Not surprisingly, much higher concentration of galactin-8 (0.75 μM), compared with VEGF-C (2.38 nM), was required to produce equivalent LEC sprouting. This is because generally, the affinity of CRD of galactins towards their glycans ligands is lower (dissociation constant: >1 μM) compared with typical protein – protein interaction (dissociation constant: ~10 nM)51. Despite the weak affinity of their CRD, galactins achieve a stable interaction with their ligands due to their multivalency that results in overall high avidity52. Therefore, even if the affinity of one galactin-8 molecule for one PDNP molecule is weak, the overall high avidity is able to activate lymphangiogenesis pathway.

Our findings that PDNP binds to galactin-8 in a carbohydrate-dependent manner, that it contains the high affinity glycans of galactin-8 (2,3-sialylated glycans), that galactin-8 clusters PDNP on cell surface, and that unlike the knockdown of VEGFR-3, knockdown of PDNP abrogates galactin-8-induced LEC sprouting suggest that PDNP is a key player in the mechanism of galactin-8-induced LEC sprouting. Another major finding of the current study is that PDNP plays a critical role in VEGF-C-mediated lymphangiogenesis. Thus far, VEGF-C- and PDNP-mediated pathways have been independently shown to promote lymphangiogenesis, but the relationship in the molecular mechanism of the two pathways has not been demonstrated. Overall, our findings suggest that a galactin-8-dependent cross-talk among VEGF-C, PDNP, and integrin pathways plays a critical role in lymphangiogenesis. This is an important conceptual advance in the understanding of the molecular mechanism of a well-known VEGF-C lymphangiogenic pathway.

Schematic representation of mechanistic aspects of galactin-8-induced lymphangiogenesis is shown in Supplementary Fig. 10. Our studies revealed that VEGFR-3 is a galactin-8-binding protein (Supplementary Fig. 4a,b), and galactin-8 clusters and retains VEGFR-3 on cell surface (Supplementary Fig. 4c). Despite this, VEGFR-3 knockout did not inhibit galactin-8-induced sprouting (Supplementary Fig. 5). This suggests that although VEGF-C-induced LEC sprouting is dependent on extracellular galactin-8, the lectin has the capacity to promote LEC sprouting independently of VEGFR-3, and VEGFR-3 may be a pseudoreceptor for galactin-8. We reason that inhibiting galactin-8 attenuates VEGF-C-mediated signalling because the function of integrins α1β1 and α5β1, rather than VEGFR-3, is inhibited by galactin-8 inhibitors. We propose that galactin-8 has a unique dual-faceted mechanism of action to promote lymphangiogenesis, where galactin-8-mediated interactions between lymphangiogenic integrins (α1β1/α5β1) and PDNP, are sufficient to activate the integrins and trigger the process of lymphangiogenesis without the involvement of VEGFR-3 (model I, Fig. 8), but in the presence of VEGF-C/VEGFR-3, PDNP-galactin-8-integrin interactions substantially increase the magnitude of lymphangiogenic pathway by potentiating the VEGF-C/VEGFR-3 signaling (model II, Fig. 8). Model I is supported by current studies showing that (i) VEGFR-3 is dispensable in galactin-8-mediated LEC sprouting; (ii) galactin-8-mediated lymphangiogenesis is dependent on PDNP and integrins α1β1/α5β1; and (iii) galactin-8 treatment increases the interaction of PDNP and integrin β1 (Supplementary Fig. 9). Model II is supported by our findings that galactin-8 potentiates VEGF-C-induced lymphangiogenesis, that galactin-8 inhibitors attenuate VEGF-C-induced lymphangiogenesis, and that galactin-8-induced LEC sprouting is reduced by α1β1 and α5β1 inhibitors (Supplementary Fig. 9).

Various studies have demonstrated the pathological contribution of lymphangiogenesis to diseases of the eye2–7. Specifically, corneal lymphatics play a vital role in the pathogenesis of graft rejection3,37,38,53–55, herpetic keratitis56, dry eye disease4, ocular allergy5 and wound healing57. In the present study, we demonstrated that: (i) a dominant negative inhibitor of galactin-8 as well as the pan inhibitor of galactins dampen lymphangiogenesis; (ii) in the mouse model of corneal allogeneic transplantation, galactin-8-induced lymphangiogenesis is associated with an increase in corneal graft rejection; and that (iii) in the mouse model of HSV keratitis, corneal pathology and lymphangiogenesis is ameliorated in galactin-8 knockout mice. These findings have broad implications for developing novel therapeutic strategies for conditions resulting from pathological lymphangiogenesis in both ocular diseases mentioned above as well as nonocular diseases such as cancer metastasis and solid organ transplant rejection. Indeed, the avascular cornea serves as an excellent in vivo model to study mechanisms of hem- and lymph-angiogenesis that are also relevant to nonocular diseases. For example, based on the findings that lymphatics in the cornea promotes graft rejection, subsequent studies reported that lymphangiogenesis also occurs in solid organ grafting, such as renal and cardiac transplantation58–60. Undeniably, corneal micropocket assays have proven to be extremely valuable in revealing the mechanism of angiogenesis in cancer and many other nonocular tissues. In conclusion, our study offers a new perspective on how glycans of the cell surface receptors can be exploited to understand and modulate the process of lymphangiogenesis.

**Methods**

**Study approval.** All animal procedures were approved by the Institutional Animal Care and Use Committee at Tufts University and were performed in accordance with the regulations of Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Vision Research and recommendations of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Tufts University Institutional Review Board/ethics committee approval was obtained for human specimen accrual for this study.

**Mice.** Lymphatic-specific Proxl-EGFP reporter mice (FVB background)61 were purchased from Mutant Mouse Regional Resource Centers, FVB/NCr mice were purchased from Charles River Laboratories, and C57BL/6 mice were purchased from Jackson Laboratory. Mice with inducible deletion of PDNP (PdpnfΔ/CagCre) and WT littermates (PdpnfΔ/+CagCre) in mixed background (C57BL/6 and 129Sv) were generated as previously described62. PDNP deletion was accomplished by administering tamoxifen orally (20 μg each day) from P1 to P6. After weaning, the mice were orally administered 1 mg tamoxifen weekly. The Lgal8K KO mouse strain used for this study was created from embryonic stem cell clone (14305A-F8), obtained from the KOMP Repository (www.komp.org) and generated by Regeneron Pharmaceuticals, Inc.63. The Lgal8 KO mice have no obvious defects in lymphatic vessel development examined by gross morphological analysis.

**Expression and purification of recombinant proteins.** All recombinant human galectins were expressed in the Escherichia coli expression system.
The recombinant galectins were induced by isopropyl β-D-1-thiogalactopyranoside (IPTG) and purified by affinity chromatography on lactosyl-sepharose beads as described previously43,45. Briefly, human galectin-1 cDNA was cloned into Ncol/Hind III cut pQE-60 (Qiagen, eliminating 6-His tag sequence residing in the 5′ end of the sequence of GST in the expression plasmid). For expression and purification of galectin-8 tagged with glutathione S-transferase (GST) (GST-galectin-8), human galectin-8 cDNA was cloned into Sall-Not cut pGEX-4-3 expression plasmid (Pharmacia Biotech) that provides the sequence of GST in the 5′ end, and the two mixtures were combined and centrifuged to remove insoluble proteins, supernatants were passed through lactosyl-sepharose beads (Sigma). Bound proteins were eluted with 150 mM lactose in PBS. Lactose was then removed by dialysis against 5% glycerol in PBS at least four times. Endotoxin was removed by Detoxi-Gel endotoxin removing gel (Thermo Scientific) and endotoxin levels were detected by ToxinSensor LAL endotoxin assay kit (GenScript). Endotoxin levels of all recombinant galectins used in this study were <0.1 EU µg⁻¹. Before use, each preparation of galectin was also tested for carbohydrate-binding activity by the red blood cell (RBC) agglutination assay. Tag-free recombinant galectin-8 (35.8 kDa, the predominant form of galectin-8) was used in this study except that GST (glutathione S-transferase)-tagged galectin-8 was used in affinity precipitation assays. To remove GST tag from GST-galectin-8, the fusion protein was incubated with human thioredoxin (Sigma, 2 µ of thioredoxin per 1 mg of fusion protein) at 25 °C for 1 h, followed by incubation with p-amino benzamidine-agarose (Sigma) to remove the added thioredoxin. Recombinant human mature VEGF-C (21 kDa) expressed in mammalian cells was purchased from Peprotech (catalog number: 100-20C).

Mouse models of corneal chemical injuries. To determine the expression pattern of galectin-8 in chemical injury, C57BL/6 mice were injured by silver nitrate and sodium hypochlorite solution as described above. The eyes were stained with immunofluorescence staining using anti-galectin-8 antibody as described above. For silver nitrate cautery, silver nitrate applicators (Grafco) were applied on the central cornea of the right eye for 5 s under a surgical microscope. The corneas were rinsed with 2 ml of PBS, and ophthalmic antibodies were topically applied to the operated eyes for 15 min. The extent of lymphangiogenesis in WT and PDPN-deficient mice, corneas were stained with eFluor 570-anti-mouse LYVE-1 (clone ALY7, eBioScience, and 1:75) and anti-CD31, clone MEC13.3, 1:100, BioLegend; Alexa Fluor 488 rat anti-mouse CD45, clone 30-F11, 1:100, BioLegend; Alexa Fluor 488 rat anti-mouse CD4, clone GK1.5, 1:100, BioLegend; Alexa Fluor 488 streptavidin, 1:300, Jackson ImmunoResearch Labs; Alexa Fluor 488 anti-mouse Ly6G, clone 1A8, 1:150, BioLegend), followed by incubation at 25 °C for 1.5 h with appropriate secondary antibodies and fluorophore-conjugated antibodies/streptavidin (Alexa Fluor 488 anti-rabbit IgG, 1:300, Invitrogen; Alexa Fluor 488 anti-rabbit IgG, 1:300, Invitrogen; Alexa Fluor 488 rat anti-mouse F4/80, clone BM8, 1:100, BioLegend; Alexa Fluor 647 rat anti-mouse CD11b, clone M1/70, 1:100, BioLegend; Alexa Fluor 647 rat anti-mouse CD45, clone 30-F11, 1:100, BioLegend; Alexa Fluor 488 anti-rat-mouse CD4, clone GR1, 1:100, BioLegend; Alexa Fluor 488 streptavidin, 1:300, Jackson ImmunoResearch Labs; Alexa Fluor 594 streptavidin, 1:300, Jackson ImmunoResearch Labs; Alexa Fluor 647 donkey anti-rat IgG, 1:300, Invitrogen). Fluorescence images were acquired by Leica TCS SPE imaging system (Leica).

Corneal mouse micropocket lymphangiogenesis assay. The corneal micropocket lymphangiogenesis assay was performed as described previously using implants containing a test agent, hydron and sucralfluoride. Test agents included full-length galectin-8 (40–320 ng per pellet) and VEGF-C (160 ng per pellet). Implants containing hydron and sucralfluoride alone were negative controls. The mice were anaesthetized by intraperitoneal injection of a cocktail of amlodipine besilate (2 mg/kg of body weight), ketamine (50 mg/kg of body weight) and xylazine (10 mg/kg of body weight). A canthotomy was performed with a von Graefe knife (Miltex), a pocket was created about 2 mm from the limbus. Using a von Graefe knife (Miltex), a pocket was created about 2 mm from the limbus. For silver nitrate cautery, silver nitrate applicators (Grafco) were applied on the corneal mouse micropocket lymphangiogenesis assay. The wound was coated with a veterinary ophthalmic ointment (Akorn) to prevent infection. Mouse corneas were harvested 7 days after pellet implantation, fixed with 4% paraformaldehyde/PBS, permeabilized with 0.3% Triton X-100/PBS, blocked with 1% BSA/PBS, and then were incubated at 4 °C overnight with primary antibodies (rabbit anti-galectin-8, NBPI-66520, 1:200, Novus; rat anti-mouse CD45, clone 30-F11, BioLegend; goat anti-type I collagen, clone D-13, 1:50, SCBT; biotinylated goat anti-mouse LYVE-1, BAF2125, 1:100, R&D Systems; goat anti-α-smooth muscle actin, clone 1A4, 1:300, BioLegend) followed by incubation at 25 °C for 1.5 h with appropriate secondary antibodies and fluorophore-conjugated antibodies/streptavidin (Alexa Fluor 488 anti-rabbit IgG, 1:300, Invitrogen; Alexa Fluor 488 anti-rabbit IgG, 1:300, Invitrogen; Alexa Fluor 488 rat anti-mouse F4/80, clone BM8, 1:100, BioLegend; Alexa Fluor 647 rat anti-mouse CD11b, clone M1/70, 1:100, BioLegend; Alexa Fluor 647 rat anti-mouse CD45, clone 30-F11, 1:100, BioLegend; Alexa Fluor 488 anti-rat-mouse CD4, clone GR1, 1:100, BioLegend; Alexa Fluor 488 streptavidin, 1:300, Jackson ImmunoResearch Labs; Alexa Fluor 594 streptavidin, 1:300, Jackson ImmunoResearch Labs; Alexa Fluor 647 donkey anti-rat IgG, 1:300, Invitrogen). Fluorescence images were acquired by Leica TCS SPE imaging system (Leica).
LEC (Lonza) were lysed in Triton lysis buffer (20 mM Tris-HCl, pH 7.4; 150 mM NaCl; 0.2–10 mM, Carbosynth), 6–sialic lactose (2–10 mM, Carbosynth), LY249002 (20 μM, Abcam), U0126 (20 μM, Abb). P30529501 (1 μM, Selleckchem), anti-integrin αv β3 functional antibody (clone 23C6, 20 μg ml–1, ebioscience), anti-integrin α5 (clone NKL-SAM-1, 20 μg ml–1, ebioscience), anti-integrin β3 functional antibody (clone KNS2, 20 μg ml–1, ebioscience), mouse IgG1 control functional grade antibody (20 μg ml–1, ebioscience), Obutstatin (2 μM, R&D Systems), and BIO121 (10 μM, R&D Systems).

Corneal transplantation. The mouse model of corneal transplantation was used as previously described. Briefly, in brief, BALB/c mice (10-week old) were used as graft recipients and male C57BL/6 mice (10-week old) were used as donors. The donor corneal grafts (2.0 mm) were sutured into 1.5 mm diameter corneal host beds of BALB/c mice recipients with 8–10 interrupted 11-0 nylon sutures. At the time of corneal transplantation, donor corneas were scarified in a grid pattern with a 30-gauge needle, and 3% Proparacaine eye drop was applied to the corneas as a topical anaesthetic. The Grafts were evaluated for signs of rejection by slit-lamp biomicroscopy (SL-D7, Nikon) and stained with Calcein AM (Invitrogen, 1 μM) or Syto11 (Molecular Probes, 1 μM) to distinguish living and dead cells.

Western blot analysis. Primary LECs were lysed with Triton lysis buffer supplemented with protease inhibitor cocktail and Phos-STOP phosphatase inhibitor cocktail (Roche), and subjected to electrophoresis in 4–15% SDS–polyacrylamide gels (Bio-Rad). Protein bands of the gels were blocked with 5 × Odyssey blocking buffer (OBB, Li-COR). For AKT and ERK signalling, bands were incubated with primary antibodies (rabbit anti-ERK1/2, 1:7,500, Cell Signaling Technology; mouse anti-phospho-ERK1,2, Thr202/Tyr204, 1:2,000, Cell Signaling Technology; monospecific anti-AKT1, 1:2,000, Cell Signaling Technology; rabbit anti-phospho-AKT, Ser473, 1:1,500, Cell Signaling Technology) overnight at 4°C. After washing with 0.5% Tween-20/PBS three times, the bands were incubated with appropriate secondary antibodies (donkey anti-rabbit IRDye 880LT and anti-rabbit IRDye 680LT, both 1:4,000, Li-COR). bands were imaged by an Odyssey Infrared Imaging System using Image Studio v2.0 software (Li-COR). For integin signalling, bands were incubated with primary antibodies (rabbit anti-phospho-integrin β1, Tyr783, 1:750, Abb; rabbit anti-phospho-integrin β1, Thr987/988, 1:750, Invitrogen; goat anti-integrin β1, N-20, 1:1,000, Santa Cruz Biotechnology; mouse anti-FAK, clone 77, 1:2,000, BD Bioscience; anti-phospho-FAK, Tyr925/927, 1:1,000, Invitrogen) overnight at 4°C. After washing with 0.5% Tween-20/PBS three times, the bands were incubated with appropriate secondary antibodies for 45 min at 25°C. The bands were scanned by an Odyssey Infrared Imaging System using Image Studio v2.0 software (Li-COR).

After scanning, the bands were stripped with NewBlot Nitrocellulose stripping buffer (25°C, 10 min, Li-Cor) and reprobed with primary antibodies (rabbit anti-VEGFR-3, clone C-20, 1:200, Santa Cruz Biotechnology; rat anti-human PDPN, 1:2,000, BioLegend; mouse anti-GAPDH, clone 6C5, 1:1,000, Santa Cruz Biotechnology; mouse anti-β-actin, clone AC-15, 1:1,000, Santa Cruz Biotechnology) overnight at 4°C. The bands were developed using appropriate secondary antibodies (goat anti-rabbit IRDye 800CW; anti-rat IRDye 800CW; anti-mouse IRDye 680LT, 1:1,000, Li-COR) for 45 min at 25°C. Signals were detected by Odyssey Infrared Imaging System.

LEC migration assay. Transwell (6.5 mm) with 8 μm-pore polycarbonate membrane inserts (Corning) were used in the migration assay. The lower side of the insert membranes were coated with 400 μl of fibronectin (10 μg ml–1, Sigma) or galectin-8 (0.5 μM) (37°C, overnight), and then the inserts were blocked with 0.1% BSA in PBS, 37°C, 3 h. The cells were then seeded onto the upper side of the insert membrane overnight, detached with StemPro Accutase cell dissociation reagent, and resuspended in serum-free EBM-2 medium (2 × 105 cells ml–1). Aliquots of LEC suspension (200 μl of 2 × 105 cells ml–1) were added to the upper chamber. The bottom chamber was filled with 600 μl of serum-free EBM-2 and the plates were incubated at 37°C for 2 h. Images were fixed at 0, 4, 8, and 24 h (25°C) and stained with Giemsa stain (40 min, 25°C) per manufacturer’s instructions. Membranes were wiped free of cells on the upper surface and mounted with Permperm mounting medium (Fisher) on glass slides. The number of migrating cells in each condition was counted in 4 random fields at 10× magnification, averaged, and normalized to control condition to generate percent-change in migration activity. In some experiments, the cells were incubated in the presence of isotype control Ab (10 μg ml–1, ebioscience) or the anti-PDPN functional blocking Ab (10 μg ml–1, ebioscience). No LECs attached to BSA-coated membranes, and therefore this group was not included in the graphs.

Immunoprecipitation. Mouse isotype antibody (15 μg, Santa Cruz Biotechnology) and anti-PDPN (15 μg, clone E-1, Santa Cruz Biotechnology) were immobilized on RockNChip Plus coupling resin (Pierce) by sodium (meth)acrylate chemistry, according to manufacturer’s instructions. Primary LECs were serum-starved overnight and treated with galectin-8 (0.2 μM) for 15 min at 37°C. After treatment, cells were lysed with IP lysis/wash buffer (25 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 5% glycerol, pH 7.4) supplemented with protease inhibitor cocktails (Roche). After centrifugation (10 min, 13,000 r.p.m.), supernatants (500 μg protein lysates) were pre-cleared by incubation for 1 h with Pierce control agarose at 4°C. The clarified samples were incubated with the Ab-conjugated agarose resins overnight at 4°C. Immunoprecipitates were washed three times with IP lysis/wash buffer and once withconditioning buffer provided by the kit (Pierce). The bound proteins were eluted with 5 × SDS–glycine electrophoresis gel (20 μg) samples were stained with Tris-HCl (pH 9.0), and analysed alongside with inputs by Western blotting using anti-integrin α1 (clone 639508, 1:500, R&D Systems), anti-integrin α5 (clone H-104, 1:1,000, Santa Cruz Biotechnology), anti-integrin β1 (clone N-20, 1:1,000, Santa Cruz Biotechnology), anti-integrin α9 (ab79795, 1:500, Abcam), anti-integrin β1 (ab87995, 1:500, Santa Cruz Biotechnology), anti-VEGFR-3 (clone H-21, 1:1,000, BD Biosciences), anti-PDPN, anti-galectin-8 (clone NB01-66520, 1:50,000, Novus Biologicals) and anti-GAPDH as described above.

Immunocytochemistry staining. Primary LECs were treated with galectin-8 (0.5 μM) for 15 min at 37°C, washed with PBS three times, and fixed with 4% paraformaldehyde/PBS (10 min, 25°C). Cells were blocked with Image-iT FX signal enhancer (30 min, 25°C, Invitrogen) and incubated with primary antibodies (mouse anti-FAK, clone 57H10, 1:200, BD Biosciences; mouse anti-GAPDH, clone 6C5, 1:1,000, Santa Cruz Biotechnology; anti-galectin-8, H-8, 1:100, Santa Cruz Biotechnology) in 2%
Flow cytometry analysis. To determine if PDNP deficiency alters cell surface expression of integrins and VEGFR-3, flow cytometry analysis was used. Primary LPCs were transfected with control siRNA or PDNP siRNA as described above. After 48 h transfection, the cells were lifted with StemPro Accutase cell dissociation reagent (Invitrogen), washed with PBS and fixed with 2% paraformaldehyde/PBS on ice for 10 min, and were then stained using rat anti-human PDNP (1:500, BioLegend), mouse anti-human VEGFR-3 (clone 9D9F9, 1:100, BioLegend), mouse anti-human integrin β1 (clone TS2/16, 1:300, BioLegend) and mouse anti-human integrin αβ (clone SNK-1AM-1, 1:300, BioLegend) primary antibodies in cell staining buffer (BioLegend, 45 min on ice) and Alexa Fluor 488 donkey anti-rat and Alexa Fluor 647 anti-mouse secondary antibodies (1:1,000, Invitrogen, 30 min on ice). For negative controls, isotype antibodies were used. The stained cells were fixed with 2% paraformaldehyde/PBS, analysed with BD FACS Calibur, and the mean fluorescence intensity of VEGFR-3 and PDNP were quantified with the FlowJo software (version 9.5.2).

Statistics. Data in all figures are presented as mean ± s.e.m. All results were confirmed in 2 or more independent experiments. Data were analysed using paired two-tailed Student’s t-test or one-way analysis of variance in Prism 6 (GraphPad) as indicated in figure legends. P value < 0.05 was considered statistically significant.

References

1. Kerjaschki, D. The lymphatic vasculature revisited. J. Clin. Invest. 124, 874–877 (2014).
2. Aspelund, A. et al. The Schlemm’s canal is a VEGF-C/VEGFR-3-responsive lymphatic-like vessel. J. Clin. Invest. 124, 3975–3986 (2014).
3. Dietrich, T. et al. Cutting edge: lymphatic vessels, not blood vessels, primarily mediate immune reactions after transplantation. J. Immunol. 184, 535–539 (2010).
4. Goyal, S., Chauhan, S. K. & Dana, R. Blockade of prolymphangiogenic vascular endothelial growth factor C in dry eye disease. Arch. Ophthalmol. 130, 84–89 (2012).
5. Lee, H. S. et al. Involvement of corneal lymphangiogenesis in a mouse model of allergic eye disease. Invest. Ophthalmol. Vis. Sci. 56, 3140–3148 (2015).
6. Thomson, B. R. et al. A lymphatic defect causes ocular hypertension and glaucoma in mice. J. Clin. Invest. 124, 4320–4324 (2014).
7. Wuest, T. R. & Carr, D. J. VEGF-A expression by HSV-1-infected cells drives corneal lymphangiogenesis. J. Exp. Med. 207, 101–115 (2010).
8. Witte, M. H. et al. Lymphangiogenesis and hemangiogenesis: potential targets for glioblastoma therapy. J. Surg. Oncol. 103, 499–500 (2011).
9. Markowska, A. I., Jefferies, K. C. & Panjwani, N. Galectin-3 protein modulates endothelial functions through interaction of VEGFR-3 and integrin alpha5beta1. J. Cell. Physiol. 202, 205–214 (2005).
10. Troncoso, M. F. et al. Galectin-8: a matricellular lectin with key roles in angiogenesis. Glycobiology 24, 907–914 (2014).
11. Kim, I. et al. Molecular cloning, expression, and characterization of angiopeptin-related protein. Glycobiology 24, 26523–26528 (1999).
12. Abdel-Malak, N. A. et al. Angiopeptin-1 promotes endothelial cell proliferation and migration through AP-1-dependent autocrine production of interleukin-8. Blood 111, 4145–4154 (2008).
13. Yoon, C. M. et al. Sphinoglycan-1 phosphate promotes lymphangiogenesis by stimulating S1P1/Gi/PLC/Ca2+ signaling pathways. Blood 112, 1129–1138 (2008).
14. Carlsson, S., Karlsson, M. C. & Leffler, H. Intracellular sorting of galectin-8 based on carbohydrate fine specificity. Glycobiology 17, 906–912 (2007).
15. Partridge, E. A. et al. Regulation of cytokine receptors by Golgi N-glycan processing and endocytosis. Science 306, 120–124 (2004).
16. Yang, R. Y., Hilt, P. N., Hsu, D. K. & Liu, T. P. Role of the carboxyl-terminal lectin domain in self-association of galectin-3. Biochemistry 37, 4896–4902 (1998).
17. Levy, Y. et al. It depends on the hinge: a structure-functional analysis of galectin-8, a tandem-repeat type lectin. Glycobiology 16, 463–476 (2006).
18. Deng, Y., Atri, D., Eichmann, A. & Simons, M. Endothelial ERK signaling controls lymphatic fate specification. J. Clin. Investig. 120, 1202–1215 (2013).
19. Zhou, F. et al. Akt/protein kinase B is required for lymphatic network formation, remodeling, and valve development. Am. J. Pathol. 177, 2124–2133 (2010).
20. Singh, N. et al. Soluble vascular endothelial growth factor receptor 3 is essential for corneal alymphaticity. Blood 121, 4242–4249 (2013).
21. Chakravorty, A. R. et al. Vascular endothelial growth factor-C promotes alloimmunity by amplifying antigen-presenting cell maturation and lymphangiogenesis. Invest. Ophthalmol. Vis. Sci. 53, 1244–1250 (2012).
22. Liesegang, T. J., Melton, 3rd L. J., Daly, P. J. & Istrup, D. M. Epidemiology of ocular herpes simplex. Incidence in Rochester, Minn, 1950 through 1982. Arch. Ophthalmol. 107, 1155–1159 (1989).
23. Park, P. J. et al. Corneal lymphangiogenesis in herpetic stromal keratitis. Surv. Ophthalmol. 60, 67–71 (2015).
24. Cursiefen, C. et al. VEGF-A stimulates lymphangiogenesis and hemangiogenesis in inflammatory neovascularization via macrophage recruitment. J. Clin. Investig. 113, 1040–1050 (2004).
25. Festa, J. et al. Alternatively spliced vascular endothelial growth factor receptor-2 is an essential endogenous inhibitor of lymphatic vessel growth. Nat. Med. 15, 1023–1030 (2009).
26. Garner, O. B. & Baum, L. G. Galectin-glycan lattices regulate cell-surface glycoprotein organization and signalling. Biochem. Soc. Trans. 36, 1472–1477 (2008).
27. Rabinovich, G. A., Toscano, M. A., Jackson, S. S. & Vasta, G. R. Functions of cell surface galectin-glycoprotein lattices. Curr. Opin. Struct. Biol. 17, 513–520 (2007).
28. Joskou, V. et al. Protein glycosylation regulates receptor specificity and activity of VEGF-C. EMBO J. 16, 3898–3911 (1997).
29. Wirzenius, M. et al. Distinct vascular endothelial growth factor signals for lymphatic vessel enlargement and sprouting. J. Exp. Med. 204, 1431–1440 (2003).
30. Vlahakis, N. E., Young, B. A., Rezaiekhaligh, M. H., Mabry, S. M. & Ekekezie, I. I. Polaredized migration of lymphatic endothelial cells is critically dependent on podoplanin regulation of Cdc42. Am. J. Physiol. Lung Cell Mol. Physiol. 300, L142 (2011).
48. Karpanen, T. et al. Functional interaction of VEGF-C and VEGF-D with neuropilin receptors. FASEB J. 20, 1462–1472 (2006).

49. Reynolds, A. R. Potential relevance of bell-shaped and u-shaped dose-responses for the therapeutic targeting of angiogenesis in cancer. Dose Response 8, 253–284 (2009).

50. Hirabayashi, J. et al. Oligosaccharide specificity of galectins: a search by frontal affinity chromatography. Biochim. Biophys. Acta 1572, 232–254 (2002).

51. Brewer, C. F., Miceli, M. C. & Baum, L. G. Clusters, bundles, arrays and lattices: novel mechanisms for lectin-saccharide-mediated cellular interactions. Curr. Opin. Struct. Biol. 12, 616–623 (2002).

52. Yamagami, S. & Dana, M. R. The critical role of lymph nodes in corneal allograft survival. Invest. Ophthalmol. Vis. Sci. 45, 1117–1124 (2004).

53. Karpanen, T.

54. Ling, S. & Dana, M. R. The critical role of lymph nodes in corneal allograft survival and graft rejection. Invest. Ophthalmol. Vis. Sci. 42, 1293–1298 (2001).

55. Emami-Naeini, P.

56. Bryant-Hudson, K. M., Gurung, H. R., Zheng, M. & Carr, D. J. Tumor necrosis factor alpha and interleukin-6 facilitate corneal lymphangiogenesis in response to herpes simplex virus 1 infection. J. Virol. 88, 14451–14457 (2014).

57. Ling, S. et al. Development of new lymphatic vessels in alkali-burned corneas. Acta Ophthalmol. 103, e315–e324 (2015).

58. Kerjaschki, D. et al. Lymphatic neoangiogenesis in human kidney transplants is associated with immunologically active lymphocytic infiltrates. J. Am. Soc. Nephrol. 15, 603–612 (2004).

59. Kerjaschki, D. et al. Lymphatic endothelial progenitor cells contribute to de novo lymphangiogenesis in human renal transplants. Nat. Med. 12, 230–234 (2006).

60. Nynskari, A. L. et al. Targeting lymphatic vessel activation and CCL21 production by vascular endothelial growth factor receptor-3 inhibition has novel immunomodulatory and antiarteriosclerotic effects in cardiac allografts. Circulation 121, 1413–1422 (2010).

61. Choi, I. et al. Visualization of lymphatic vessels by Proxl-promoter directed GFP reporter in a bacterial artificial chromosome-based transgenic mouse. Blood 117, 362–365 (2011).

62. Valenzuela, D. M. et al. High-throughput engineering of the mouse genome coupled with high-resolution expression analysis. Nat. Biotechnol. 21, 652–659 (2003).

63. Poland, P. A., Kirlough, C. L. & Hughey, R. P. Cloning, expression, and purification of galectins for in vitro studies. Methods Mol. Biol. 1207, 37–49 (2015).

64. Paranthan, R. R., Bargagna-Mohan, P., Lau, D. L. & Mohan, R. A robust model for simultaneously inducing corneal neovascularization and retinal gliosis in the mouse eye. Mol. Vis. 17, 1901–1908 (2011).

65. Panjwani, N., Moulton, P., Alroy, J. & Baum, I. Localization of lectin binding sites in human, cat, and rabbit corneas. Invest. Ophthalmol. Vis. Sci. 27, 1280–1284 (1986).

66. Cao, R. et al. Mouse corneal lymphangiogenesis model. Nat. Protoc. 6, 817–826 (2011).

67. Rogers, M. S., Birzner, A. E. & D’Amato, R. J. The mouse cornea micropocket angiogenesis assay. Nat. Protoc. 2, 2545–2550 (2007).

68. Sugaya, S. et al. Comparison of galectin expression signatures in rejected and accepted murine corneal allografts. Cornea 34, 675–681 (2015).

69. Sano, Y., Kasander, B. R. & Streilein, J. W. Minor H, rather than MHC, alloantigens offer the greater barrier to successful orthotopic corneal transplantation in mice. Transpl. Immunol. 4, 53–56 (1996).

70. Cursiefen, C. et al. Roles of thrombospondin-1 and -2 in regulating corneal and iris angiogenesis. Invest. Ophthalmol. Vis. Sci. 45, 1117–1125 (2004).

Acknowledgements
We thank Lucy Liaw (Maine Medical Center Research Institute, MMCRI), Peter Brooks (MMCRI), John Castellot (Tufts University) and Pablo Argueso (Schepps Eye Institute) for helpful discussions. We also thank Hsuan-Chun Lin (Case Western Reserve University) and Albert Tai (Tufts Genomics Core) for their expert help and guidance in kinetics studies and RNA Seq analysis, respectively. This work was supported by National Eye Institute Grant R01EY007088 and R01EY009349 to NP; R01EY026265 to PF; Mass Lions Eye Research Fund, New England Corneal Transplant Fund, an unrestricted award from Research to Prevent Blindness to the Department of Ophthalmology, Tufts University; Tufts Medical Center institutional support; and P30NS047243 to Tufts Imaging Core at Center for Neuroscience Research.

Author contributions
Chen, W.-S.C. and N.P. designed the research, analysed data, and wrote the manuscript; W.-S.C, Z.C., S.S., M.L. and V.G.S. performed research, analysed data, and contributed to manuscript writing; and N.L., H.L., U.J.N., J.F., J.S., L.X. and P.H. contributed to research design and manuscript writing.

Additional information
Accession codes: RNA-seq data have been deposited in the Gene Expression Omnibus (GEO) database under accession code GSE79160.

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: The authors declare no competing financial interests.

Reprints and permission information is available online at http://npg.nature.com/reprintsandpermissions.

How to cite this article: Chen, W.-S. et al. Pathological lymphangiogenesis is modulated by galectin-8-dependent crosstalk between podoplanin and integrin-associated VEGFR-3. Nat. Commun. 7:11302 doi: 10.1038/ncomms11302 (2016).
Erratum: Pathological lymphangiogenesis is modulated by galectin-8-dependent crosstalk between podoplanin and integrin-associated VEGFR-3

Wei-Sheng Chen, Zhiyi Cao, Satoshi Sugaya, Maria J. Lopez, Victor G. Sendra, Nora Laver, Hakon Leffler, Ulf J. Nilsson, Jianxin Fu, Jianhua Song, Lijun Xia, Pedram Hamrah & Noorjahan Panjwani

Nature Communications 7:11302 doi: 10.1038/ncomms11302 (2016); Published 12 Apr 2016; Updated 19 Jul 2016

In Fig. 6a of this Article the sixth histogram bars were accidentally mislabeled during the production process. The label ‘VEGF-C + anti-β3’ should read ‘VEGF-C + anti-β5’, and ‘Gal-8 + anti-β3’ should read ‘Gal-8 + anti-β5’.

The correct version of the figure appears below.
Figure 6

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/