Thrombospondin 1 inhibits inflammatory lymphangiogenesis by CD36 ligation on monocytes

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Lymphangiogenesis plays an important role in tumor metastasis and transplant outcome. Here, we show that thrombospondin-1 (TSP-1), a multifunctional extracellular matrix protein and naturally occurring inhibitor of angiogenesis inhibits lymphangiogenesis in mice. Compared with wild-type mice, 6-mo-old TSP-1–deficient mice develop increased spontaneous corneal lymph-angiogenesis. Similarly, in a model of inflammation-induced corneal neovascularization, young TSP-1–deficient mice develop exacerbated lymphangiogenesis, which can be reversed by topical application of recombinant human TSP-1. Such increased corneal lymphangiogenesis is also detected in mice lacking CD36, a receptor for TSP-1. In these mice, repopulation of corneal macrophages with predominantly WT mice via bone marrow reconstitution ameliorates their prolymphangiogenic phenotype. In vitro, exposure of WT macrophages to TSP-1 suppresses expression of lymphangiogenic factors vascular endothelial growth factor (VEGF)-C and VEGF-D, but not of a primarily hemangiogenic factor VEGF-A. Inhibition of VEGF-C is not detected in the absence or blockade of CD36. These findings suggest that TSP-1, by ligating CD36 on monocytic cells, acts as an endogenous inhibitor of lymphangiogenesis.

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Abbreviations used: LYVE-1, lymphatic vessel endothelial hyaluronan receptor-1; TSP-1, thrombospondin-1; VEGF, vascular endothelial growth factor.

The outgrowth of new from preexisting lymphatic vessels (lymphangiogenesis) has recently gained wide attention for its involvement in tumor metastasis and induction of alloimmunity after transplantation (Stacker et al., 2002; Chen et al., 2004; Cursiefen et al., 2004a; Kerjaschki, 2006; Kerjaschki et al., 2006). Lymphangiogenesis is mainly induced by ligation of vascular endothelial growth factor (VEGF)-C and VEGF-D to their high-affinity receptor VEGFR3 on lymphatic vascular endothelium (Carmeliet and Jain, 2000; Stacker et al., 2002). Additionally, fibroblast growth factor (FGF) and VEGF-A have been reported to support lymphangiogenesis (Kubo et al., 2002; Nagy et al., 2002; Cursiefen et al., 2004b; Bock et al., 2007). Exogenous pharmacological inhibitors of lymphangiogenesis include corticosteroids (Boneham and Collin, 1995), VEGF-A–specific cytokine traps (VEGF Trap$_{R1R2}$; Cursiefen et al., 2004a), inhibitory peptides against integrin α5/v, and blocking antibodies against VEGF receptor 3 (Cursiefen et al., 2005; Dietrich et al., 2007; Bock et al., 2008) or VEGF-A (Bock et al., 2007). Although several lymphangiogenic growth factors and their exogenous inhibitors are already characterized, only one endogenous inhibitor of lymphatic outgrowth has been identified thus far (soluble VEGFR2; Albuquerque et al., 2009).

The avascular nature of the cornea is attributed to the presence of several antihemangiogenic factors, such as thrombospondin-1 (TSP-1) and TSP-2, angiostatin, endostatin, pigment epithelium–derived factor, and IL-1 receptor antagonist (Chang et al., 2001), which are believed to maintain the hemangiogenic privilege in this tissue. Besides these factors, receptor decoy mechanisms such as ectopically expressed

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VEGFR3 and soluble VEGFR1 (Ambati et al., 2006; Cursiefen et al., 2006) also help to maintain the avascular status of the cornea. Similarly, it is quite possible that the alymphatic nature of the cornea is also maintained by multiple endogenously expressed factors with antilymphangiogenic properties besides soluble VEGFR2 (Albuquerque et al., 2009). Knowledge about endogenous inhibitors of lymphangiogenesis not only facilitates a better understanding of the regulation of lymphangiogenesis in vivo but also explains the alymphatic nature of certain tissues, such as cartilage and cornea. Considering the potential therapeutic benefit (e.g., in tumor metastasis and transplantation immunology) of such inhibitors, we were interested in identifying additional endogenous antilymphangiogenic factors.

Many exogenous antihemangiogenic factors have been recently demonstrated to exhibit antilymphangiogenic properties (Bock et al., 2007). Thrombospondin-1 and -2 constitute an antiangiogenic subfamily (Armstrong and Bornstein, 2003; Lawler, 2000, 2002) of matricellular glycoproteins known as thrombospondins. Both isoforms are constitutively expressed in the mouse cornea (Armstrong and Bornstein, 2003; Cursiefen et al., 2004c; Sekiyama et al., 2006). TSP-1, which binds and activates latent TGF-β, inhibits hemangiogenesis through both direct effect on endothelial cell migration and survival (e.g., by inducing vascular endothelial cell apoptosis through binding to CD36; Jiménez et al., 2000), as well as through indirect effects on growth factor mobilization (e.g., by binding heparin sulfate proteoglycans [Lawler, 2000, 2002]). In addition it was shown that TSP-1 inhibits corneal hemangiogenesis in vivo (Simantov et al., 2001; Cursiefen et al., 2004c). Here, we evaluated the potential antilymphangiogenic role of TSP-1 using the mouse model of concurrent induction of hemangiogenesis and lymphangiogenesis into the normally avascular cornea (Cursiefen et al., 2004b). We provide evidence that TSP-1 indeed inhibits lymphangiogenesis and effectively regulates the expression of the main lymphangiogenic growth factor VEGF-C in inflammatory macrophages. The antilymphangiogenic effect of TSP-1 under inflammatory conditions seems to be mediated indirectly via down-regulation of lymphangiogenic growth factor VEGF-C expression in bone marrow–derived macrophages through CD36 ligation. This opens new treatment avenues for the modulation of inflammatory pathological lymphangiogenesis in the context of tumor growth and transplantation.

RESULTS

Increased lymphangiogenesis is detectable in TSP-1$^{-/-}$ mice

To analyze a potential antilymphangiogenic effect of TSP-1, we first evaluated corneal lymphangiogenesis in TSP-1$^{-/-}$ mice. Corneas of TSP-1$^{-/-}$ and WT C57BL/6 control mice were examined biomicroscopically and immunohistochemically for the lymphatic vessel marker LYVE-1 and pan-endothelial cell marker CD31. Although the corneas did not display any biomicroscopic evidence of blood vessel growth into the cornea, mild (clinically invisible) spontaneous growth of lymphatic vessels was detectable by specific immunostaining. Isolated outgrowth of LYVE-1^{+++}/CD31^{+} lymphatic vessels was detectable beyond the limbal vascular arcade into the normally avascular and alymphatic central cornea in 6-mo-old TSP-1$^{-/-}$ mice (Fig. 1, A–D). Occasionally, these lymphatic

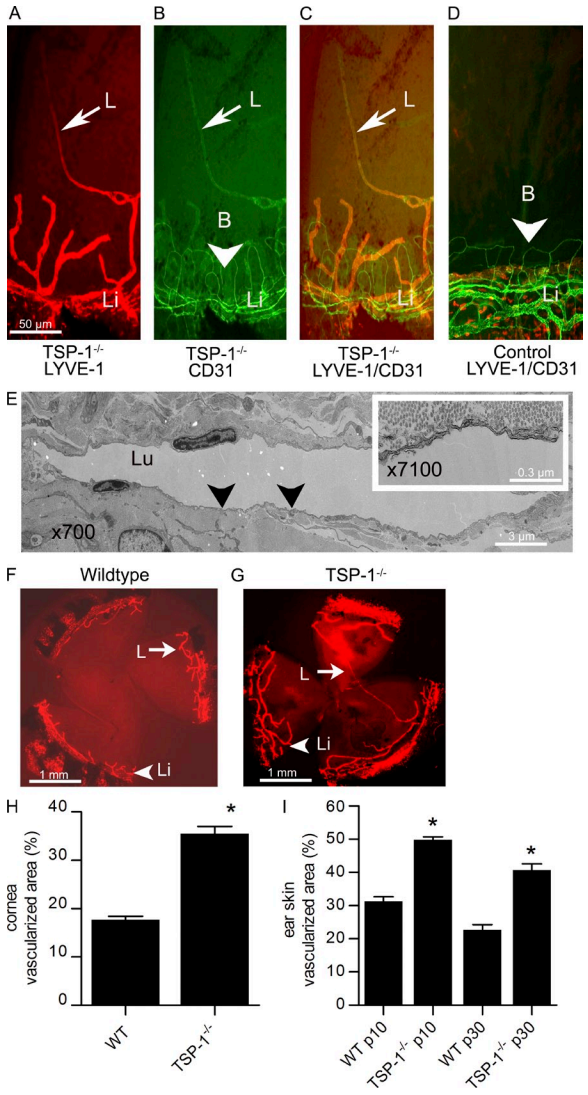

Figure 1. TSP-1$^{-/-}$ mice display mild isolated lymphangiogenesis. (A–D) Immunofluorescence analysis of LYVE-1^{+++}/CD31^{+} lymphatic vessels (L, arrows) emanating from the limbal arcade (Li) toward the center of the cornea in 6-mo-old TSP-1$^{-/-}$ (A-C) or WT (control, D) mice. Blood vessels (B, arrowheads) in the limbal arcade stain intensely with anti-CD31, but not with anti–LYVE-1. (E) Electron micrograph of the corneal stromal lymphatic vessel in TSP-1$^{-/-}$ mice showing erythrocyte-free lumen (Lu). Arrowheads denote the thin endothelial lining with absent basement membrane and absent pericyte coverage. Inset shows higher magnification view. (F and G) WT control and TSP-1$^{-/-}$ complete corneal flat-mounts stained with LYVE-1 (Li, limbus, arrowhead; L, lymphatic vessel, arrow). Note isolated lymphatic vessel traversing the whole cornea in G (L, arrow). (H) Morphometric comparison demonstrates a significantly increased spontaneous corneal lymphangiogenesis in TSP-1$^{-/-}$ mice compared with WT controls (*, $P < 0.05$; $n = 10$; all mice aged 6 mo). (I) Morphometric quantification of lymphatic vessels in the ear skin from young (p10) or adult (p30) WT and TSP-1$^{-/-}$ mice (*, $P < 0.05$; $n = 5$ per group, compared with WT controls). Error bars indicate SD.

vessels were found extending from the limbus through the corneal apex to the other side (Fig. 1 G). These LYVE-1^{+++}/CD31^{+} lymphatic vessels were not accompanied by CD31^{+++}/LYVE-1^{-} blood vessels (Fig. 1 C). Transmission electron microscopy of the central cornea confirmed the immunohistochemical findings by demonstrating lymph vessels with typical ultrastructural features (Fig. 1 E; Cursiefen et al., 2002). Morphometric comparison of the area covered by LYVE-1^{+++}/CD31^{+} lymphatic vessels in TSP-1$^{-/-}$ and age-matched WT control corneas (Fig. 1 H) revealed that the area covered by lymphatic vessels was significantly larger in the TSP-1$^{-/-}$ corneas as compared with that in WT control mice ($P < 0.001$; $n = 10$).

To determine whether the antilymphangiogenic effect of TSP-1 is detectable in tissues other than cornea, we evaluated the lymphatic vessels in typically highly lymphvascularized tissue, such as skin. Comparing the ear skins of TSP-1$^{-/-}$ and age-matched WT mice, we noted a significant increase of lymphatic vessel density in adult (p30) and young (p10) TSP-1$^{-/-}$ mice ($P < 0.05$; Fig. 1 I), suggesting that TSP-1 has antilymphangiogenic effects in different tissues.

Because spontaneous corneal lymphangiogenesis was detectable only in older (6-mo-old) TSP $^{-/-}$ mice, we next examined if corneas in younger animals exposed to inflammatory stimuli respond with increased lymphangiogenesis. We used the mouse model of suture-induced, inflammation-associated corneal neovascularization (Cursiefen et al., 2004a,b). 1 wk after intrastromal placement of three 11–0 nylon sutures in TSP-1$^{-/-}$ and WT mice, corneas were excised and the lymphvascularized area was measured using morphometry. As seen in Fig. 2 C, a significant increase in lymphangiogenesis was detected in TSP-1$^{-/-}$ mice compared with WT controls ($P < 0.05$; $n = 10$). In addition, we analyzed the effect of exogenous recombinant TSP-1 on lymphangiogenesis in vivo. WT mice received a subconjunctival injection of recombinant TSP-1 (50 ng/50 µl) before suture placement, followed by a topical treatment with TSP-1 eye drops for 7 d and two more subconjunctival injections on day 3 and 5. 1 wk after intrastromal suture placement, corneas were excised and the hemvascularized and lymphvascularized area measured using morphometry. As shown in Fig. 2 D, lymphangiogenesis was significantly inhibited (58.3 ± 12.8%; $P < 0.05$). In addition, blood vessel outgrowth was impaired significantly (81.4 ± 5.7%; $P < 0.05$; unpublished data) as expected. These findings confirm an inhibitory effect of TSP-1 in inflammation-associated lymphangiogenesis.

Expression of lymphangiogenic factor VEGF-C is increased in TSP-1$^{-/-}$ corneas

Lymphangiogenic effects of VEGF-C have been reported previously (Enholm et al., 2001; Kubo et al., 2002; Stacker et al., 2002). Binding of this growth factor to its receptor (VEGFR3) expressed on lymphatic endothelium is known to induce lymphangiogenesis. It is therefore conceivable that interference with this receptor ligand interaction may result in inhibition of lymphangiogenesis. To test whether the antilymphangiogenic effect of TSP-1 occurs via interfering with VEGF-C–mediated mechanism, we compared transcription levels of VEGF-C mRNA in corneas derived from WT and age-matched TSP-1$^{-/-}$ mice (pooled from $n = 5–8$) of different ages (8 wk and 6 mo). As depicted in Fig. 3 A, the corneas of younger TSP-1$^{-/-}$ mice did not express significantly increased mRNA levels for VEGF-C compared with WT controls. However, at 6 mo of age VEGF-C expression in TSP-1$^{-/-}$ corneas increased to more than eightfold compared with age-matched WT controls (Fig. 3 B). These results are consistent with the absence of spontaneous corneal lymphangiogenesis in young TSP-1$^{-/-}$ mice and increase detected in older mice.

Under inflammatory conditions, macrophages are known to be the primary source of the lymphangiogenic factor VEGF-C (Schoppmann et al., 2002; Cursiefen et al., 2004b; Kerjaschki, 2005; Maruyama et al., 2005, 2007). Moreover, the corneal stroma is endowed with significant numbers of resident macrophages (Brissette-Storkus et al., 2002). Therefore we examined by flow cytometry numbers of CD11b^{+} macrophages in corneas harvested from 6-mo-old WT and TSP-1$^{-/-}$ mice. The increased numbers of CD11b^{+} macrophages detectable in TSP-1$^{-/-}$ corneas (Fig. 3 C) coincided with the increased expression of corneal VEGF-C at this age compared with WT controls (Fig. 3 B). These data suggest that the absence of TSP-1 results in increased expression of the lymphangiogenic

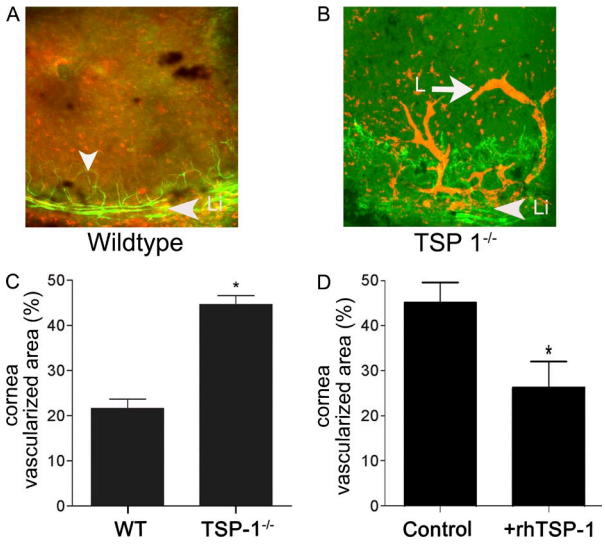

Figure 2. TSP-1 regulates the lymphangiogenic response in a mouse model of inflammation-associated corneal neovascularization. Representative segments of corneal flat mounts from WT and TSP-1$^{-/-}$ mice stained with CD31 are shown in green (blood vessels) and LYVE-1 is shown in red (lymphatic vessels; A; Li, Limbal vascular arcade: bottom, center of the cornea: top; B; L, lymph vessels). (C) Morphometric quantification of lymphvascularized area in WT and TSP-1$^{-/-}$ mice (*, $P < 0.05$; $n = 10$ per group; age 8 wk). (D) Morphometric analysis of vascularized areas with lymphatic vessels in sutured corneas of WT mice treated with three subconjunctival injections and topical eye drops (x3 daily) of recombinant human (rh) TSP-1 in comparison to control (*, $P < 0.05$; $n = 8$ per group, age 8 wk). Error bars indicate SD.

factor VEGF-C in the adult mouse cornea and corresponds with the increased numbers of macrophages. This observation is consistent with the noted increase in corneal lymphvascular areas in older TSP-1$^{-/-}$ mice. Furthermore, macrophages infiltrating the peritoneal cavity in response to an inflammatory stimulus such as thioglycollate, harvested from young TSP-1$^{-/-}$ mice, expressed significantly increased levels of VEGF-C compared with those derived from age-matched WT mice (Fig. 3 D). These results further support the increased lymphangiogenesis detectable in younger TSP-1$^{-/-}$ mice in response to suture-induced inflammation. Together, these results strongly suggest that TSP-1 exerts its antilymphangiogenic effect by reducing tissue levels of lymphangiogenic VEGF-C presumably by regulating its secretion from macrophages.

Increased inflammation-induced lymphangiogenesis in CD36$^{-/-}$ mice

The coincident increase in macrophages and VEGF-C in TSP-1$^{-/-}$ corneas points to the possibility that these corneal macrophages are involved in bringing about the antilymphangiogenic effect of TSP-1. In other words, TSP-1 may influence the ability of resident macrophages to secrete VEGF-C and regulate lymphangiogenesis. To determine if expression of VEGF-C by corneal macrophages may be regulated via their receptor CD36, which is a well-known receptor for TSP-1 (Lawler, 2000, 2002; Armstrong and Bornstein, 2003), we next evaluated whether corneas from CD36$^{-/-}$ mice resembled those from TSP-1$^{-/-}$ mice with respect to the increased lymphvascular area under inflammatory conditions. We examined suture-induced, inflammation-associated corneal lymphangiogenesis in CD36$^{-/-}$ mice. As seen in Fig. 4, we found a significantly greater degree of lymphangiogenesis in CD36$^{-/-}$ mice compared with age-matched WT controls. These results do resemble those seen in TSP-1$^{-/-}$ mice. Therefore, these data are also supportive of an indirect inhibitory effect exerted by TSP-1 on lymphangiogenesis involving CD36-mediated signaling events in macrophages.

The TSP-1 receptor CD36 is expressed on corneal macrophages, but not significantly on lymphatic vessels

Blood vascular endothelial cells are known to strongly express CD36 and ligation of this receptor by TSP-1 has been previously reported to induce apoptosis in these cells (Jiménez et al., 2000; Lawler, 2000, 2002; Armstrong and Bornstein, 2003). Thus, the antihemangiogenic effect of TSP-1 is mediated via CD36 on vascular endothelium. Based on this concept, we analyzed whether TSP-1 might exert its antilymphangiogenic effect via CD36 on lymphatic endothelial cells. To do this, we examined the expression of CD36 in the cornea and especially on resting limbal lymphatic vessels. As depicted in Fig. 5 A, there was a strong expression of CD36 on resident CD11b^{+} corneal macrophages. However, there was no obvious colocalization of CD36 with LYVE-1^{+++} lymphatic vessels (Fig. 5 B). This finding is consistent with previous studies that were unable to demonstrate a significant expression of

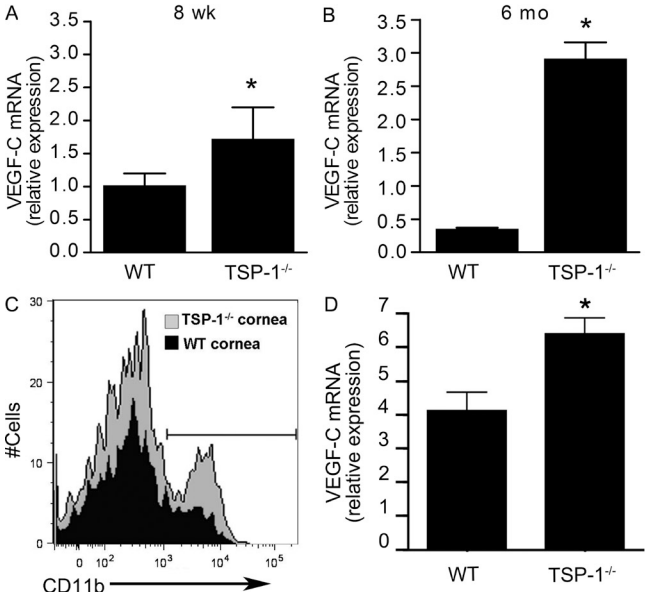

Figure 3. Increased transcription of the lymphangiogenic growth factor VEGF-C in TSP-1$^{-/-}$ mice. VEGF-C mRNA levels in corneas of 8-wk-old (A) and 6-mo-old (B) TSP-1$^{-/-}$ mice in comparison to WT controls. (C) Flow cytometric detection of CD11b^{+} macrophages in the corneas harvested from 6-mo-old TSP-1$^{-/-}$ mice (24.6%) compared with WT controls (19.7%). (D) Expression of VEGF-C in thioglycollate-elicited macrophages from TSP-1$^{-/-}$ mice (age 8 wk) compared with WT mice detected by real-time PCR. Corneas were pooled from five mice per group in each experiment. Expression of VEGF-C relative to the GAPDH in four PCR reactions per RNA sample is indicated. *, $P < 0.05$ compared with WT control. Error bars indicate SD.

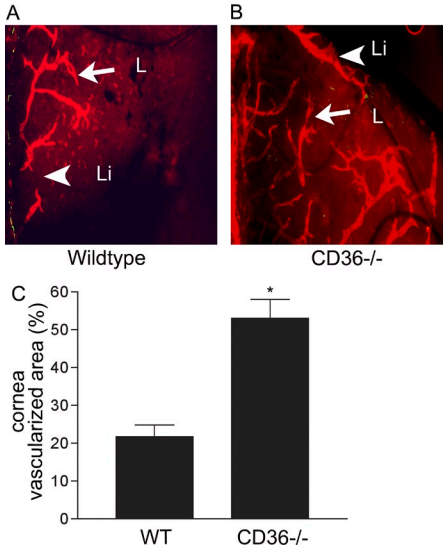

Figure 4. Increased inflammation-induced lymphangiogenesis in CD36$^{-/-}$ mice. Representative segments of corneal flat mounts from WT (A) and a CD36$^{-/-}$ (B) mice. (C) Morphometric comparison of lymphvascularized area between WT control and CD36$^{-/-}$ mice (*, $P < 0.05$; $n = 10$ per group; age 8 wk). Li, limbal vascular arcade bottom; center of the cornea, top; flat mount stained with LYVE-1, red (lymphatic vessels); L, lymph vessels. Error bars indicate SD.

CD36 in skin lymphatic vessels (Hawighorst et al., 2002). The absence of CD36 on corneal lymphatic vessels further supports an indirect antilymphangiogenic effect of TSP-1 via its ligation on resident macrophages.

Reconstitution of CD36$^{-/-}$ mice with CD36$^{+/+}$ bone marrow reverses the prolymphangiogenic phenotype of CD36$^{-/-}$ mice

Our results so far point to the CD36 receptor as a potential mediator of antilymphangiogenic effect of TSP-1. Expression of this receptor on resident corneal macrophages suggests these cells as likely candidates involved in bringing about the indirect effect of TSP-1 on lymphangiogenesis. Macrophages are bone marrow–derived cells and are potent amplifiers of lymphangiogenic signals during inflammation (Cursiefen et al., 2004b; Kerjaschki, 2005; Maruyama et al., 2007). To evaluate their role in corneal lymphangiogenesis, we first irradiated CD36$^{-/-}$ mice and reconstituted them with normal WT bone marrow cells. As a control, irradiated WT mice were also reconstituted with WT bone marrow cells. We then compared the degree of hemangiogenesis and lymphangiogenesis in these animals in response to suture-induced inflammation. At 8 wk after bone marrow reconstitution, no significant difference was detectable between CD36$^{-/-}$ mice reconstituted with WT bone marrow and the control mice in the degree of hemangiogenesis ($P > 0.05$; NS) and lymphangiogenesis ($P > 0.05$; NS; Fig. 5 C). These results clearly indicate that reconstitution with WT bone marrow that presumably restored cornea with CD36-expressing macrophages reversed the prolymphangiogenic corneal environment in CD36$^{-/-}$ mice. Our results therefore confirm an indirect inhibitory effect of TSP-1 on corneal inflammation–associated lymphangiogenesis via CD36 expressed on local macrophages.

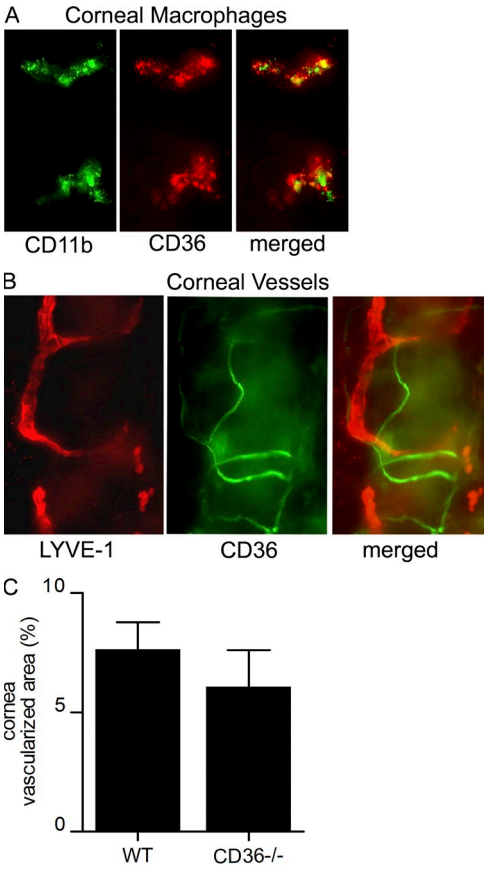

Figure 5. CD36 on macrophages regulates the lymphangiogenic response in the cornea. (A) The macrophage marker CD11b (green) and the TSP-1 receptor CD36 (red) are colocalized (yellow) on monocytic cells in the normal corneal stroma. (B) The specific lymphatic marker LYVE-1 (red) is not colocalized with antibodies against CD36 (green), and is expressed by blood vessels detectable at the limbus. (C) Morphometric comparison of corneal lymphvascularized area between WT ($n = 8$) control and CD36$^{-/-}$ ($n = 9$) mice (reconstituted with WT bone marrow) in a mouse model of inflammatory corneal neovascularization. Error bars indicate SD.

Expression of VEGF-C in macrophages is regulated by TSP-1 via CD36

It is well established that binding of TSP-1 to CD36 on macrophages is a prerequisite to the activation of latent TGFβ (Yehualaeshet et al., 1999, 2000; Chen et al., 2009a,b; Wang et al., 2010). To determine if this TSP-1–CD36 interaction can inhibit VEGF-C expression in macrophages, we first compared VEGF-C expression in thioglycollate-elicited macrophages derived from WT and CD36$^{-/-}$ mice. A significantly reduced expression of VEGF-C in the latter (Fig. 6 A; $P < 0.05$) correlates with their previously reported reduced ability to generate biologically active TGFβ (Yehualaeshet et al., 1999) and to induce VEGF-C via TGFβRI ligation in peritoneal macrophages (Oka et al., 2008). Consistent with these studies, exogenously provided TGFβ successfully increases expression of VEGF-C in CD36$^{-/-}$ macrophages as detected by increased message as well as the protein level compared with WT control cells (Fig. 6, B and C; $P < 0.05$). We next treated WT or CD36$^{-/-}$ macrophages with TSP-1 and as shown in Fig. 6 D. Although inhibition of VEGF-C was detectable in WT macrophages, no such inhibition was detected in CD36$^{-/-}$ macrophages ($P < 0.05$).

To further confirm that the inhibitory effect of TSP-1 on VEGF-C expression is indeed delivered via CD36, we used a TSP-1–derived antagonistic peptide that binds CD36 and can block further binding of TSP-1 to this receptor. As shown in Fig. 6 E the inhibitory effect of TSP on the expression of VEGF-C by WT macrophages was not affected by the control peptide. In contrast, the CD36-blocking peptide partially, albeit significantly, reversed this inhibition.

Similar to VEGF-C, the expression of VEGF-D was inhibited by TSP-1 in WT macrophages (Fig. 6 F), whereas VEGF-A expression remained unaltered (Fig. 6 G).

These results clearly demonstrate that the ligation of TSP-1 to CD36 on macrophages prevents their expression of lymphangiogenic factors, and thus explains the indirect inhibitory effect of TSP-1 on corneal lymphangiogenesis.

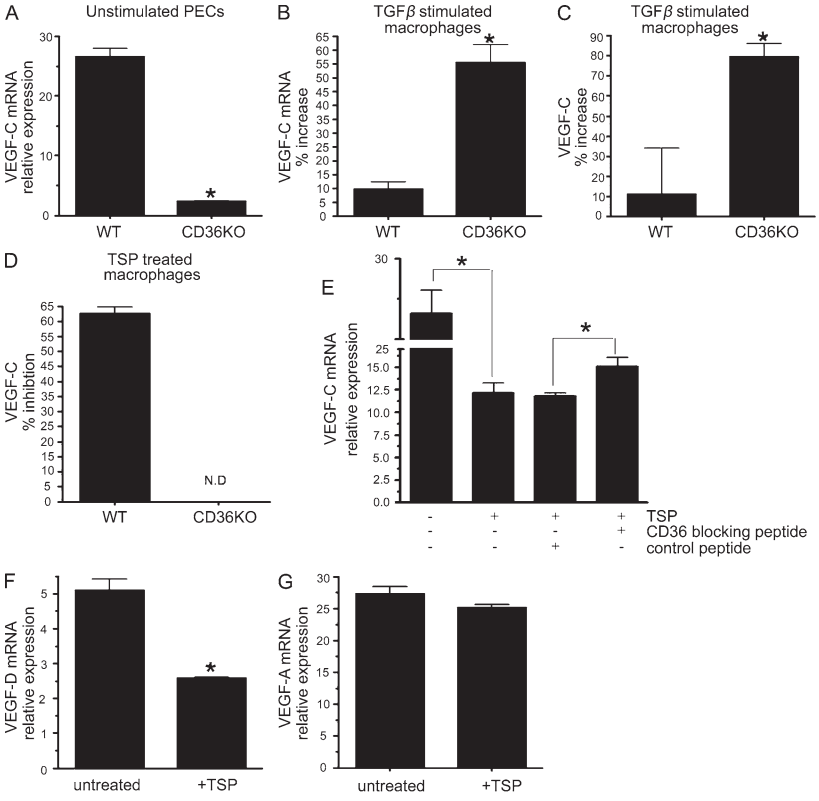

Figure 6. TSP-1 down-regulates expression of VEGF-C in macrophages via CD36. Relative expression of VEGF-C (A, B, D, and E), VEGF-D (F), and VEGF-A (G) message and VEGF-C protein (C) in thioglycollate-elicited macrophages from WT or CD36$^{-/-}$ mice: (A) freshly harvested, (B–G) cultured in the absence or presence of TGFβ2, TSP-1, or CD36 blocking and control peptides. Total RNA harvested from macrophages was subjected to real-time PCR analysis to detect expression of VEGF-C, VEGF-D, and VEGF-A mRNA and to detect VEGF-C protein culture supernatants were analyzed by ELISA. Although freshly harvested CD36$^{-/-}$ macrophages express significantly lower levels of VEGF-C compared with WT controls (A) these macrophages retain their ability to respond to exogenously provided TGFβ2 in culture by increasing expression of VEGF-C (both message [B] and protein [C]) compared with WT controls. Inhibition of VEGF-C expression detectable in WT macrophages cultured with TSP-1 (5 µg/ml) is not detected in similarly treated CD36 $^{-/-}$ macrophages (D). In the presence of CD36-binding TSP-1–derived peptide capable of blocking CD36–TSP-1 interaction (CD36 blocking peptide), the inhibitory effect of TSP-1 on the VEGF-C expression in WT macrophages is significantly reversed (E). Treatment of WT macrophages to TSP-1 also inhibited the expression of the lymphangiogenesis factor VEGF-D (F), but not that of predominantly hemangiogenic factor VEGF-A (G). Expression of VEGF-C, VEGF-D, and VEGF-A relative to the GAPDH in four independent PCR reactions per RNA sample is indicated. *, $P < 0.05$ compared with WT, untreated or indicated control. Error bars indicate SD.

DISCUSSION

In this study, we demonstrate for the first time that TSP-1 plays a crucial role in the regulation of inflammatory lymphangiogenesis. We show that this multifunctional matrix protein regulates the expression of VEGF-C at the mRNA level in macrophages. In addition, we were able to demonstrate that this regulation is mediated via the receptor CD36. Using a model of inflammatory lymphangiogenesis in an otherwise angiogenically privileged site, such as the cornea, we demonstrate that the absence of TSP-1 leads to a significant increase in lymphangiogenesis. This observation underscores the importance of TSP-1 in maintaining the avascular status of the immune-privileged corneal tissue. Our findings are further supported by the observation that 6-mo-old TSP$^{-/-}$ mice develop spontaneous corneal lymphangiogenesis and, in parallel, exhibit a massive increase in the lymphangiogenic growth factor VEGF-C in comparison to young animals.

Whereas the pro- and antihemangiogenic factors balancing the process of blood vessel outgrowth (hemangiogenesis) have received much attention over the past years with a large array of known factors (Folkman, 1995; Carmeliet and Jain, 2000; Chang et al., 2001), less is known about the factors involved in the induction and prevention of lymphatic vessels (lymphangiogenesis). Both the VEGF family (VEGF-A, -C, and -D; Folkman, 1995; Carmeliet and Jain, 2000; Enholm et al., 2001; Veikkola et al., 2001; Nagy et al., 2002; Podgrabinska et al., 2002; Saaristo et al., 2002; Stacker et al., 2002; Rissanen et al., 2003; Cursiefen et al., 2004b) and bFGF can induce the outgrowth of lymphatic vessels (Kubo et al., 2002). In contrast, it remains unclear how the ingrowth of lymphatic vessels (into alymphatic tissues) is prevented. The cornea is normally devoid of both blood and lymphatic vessels, but can be invaded by both vessel types after severe inflammatory insults (Cursiefen et al., 2002; Kubo et al., 2002). Using the normally alymphatic cornea as a model, we identify TSP-1 as a novel endogenous inhibitor of lymphangiogenesis. The normal cornea contains both message and protein for TSP-1 (Cursiefen et al., 2004c), especially in the inner and outer layers (basal epithelium and Descemet's membrane; Hiscott et al., 1997). Two of our findings suggest that TSP-1 is likely to be one of several antilymphangiogenic factors within the cornea: (1) there was only mild spontaneous lymphangiogenesis in TSP-1$^{-/-}$ mice and (2) the effect was only observed in older mice. Therefore, other mechanisms such as the recently identified role of a splice variant of sVEGFR2 in maintaining corneal alymphatic status (Albuquerque et al., 2009) might be at work in TSP-1$^{-/-}$ mice. Furthermore, based on increased lymphatic vessels detectable in the TSP-1$^{-/-}$ cutaneous tissue, we conclude that the inhibitory effect of TSP-1 on lymphangiogenesis is also applicable in nonimmune privileged sites.

Although mildly increased corneal lymphvascular area was detectable in older CD36$^{-/-}$ mice (unpublished data), in younger mice a profound increase in corneal lymphangiogenesis was noted in an inflammatory milieu similar to that seen in TSP-1$^{-/-}$ mice. These results implicated CD36 as a

potential TSP-1 receptor likely to regulate lymphangiogenesis. Because of the absence of CD36 on lymphatic vessels (Hawighorst et al., 2002), unlike on blood vessels, we demonstrate that the antilymphangiogenic effect of TSP-1 in the cornea is mediated indirectly via CD36-expressing local macrophages. Under inflammatory conditions, corneal macrophages are known to contribute to lymphangiogenesis, presumably by serving as a source of VEGF-C (Maruyama et al., 2005). In TSP-1$^{-/-}$ corneas, increased expression of VEGF-C was accompanied with an increased numbers of macrophages. These results are consistent with an increased expression of a macrophage chemotactic factor, MCP-1, reported to be one of the inflammatory mediators expressed in corneas derived from older TSP-1$^{-/-}$ mice (Turpie et al., 2009). These mice were reported to spontaneously develop ocular surface inflammation with increasing age. Finally, the reversal of inflammation induced corneal lymphangiogenesis in CD36$^{-/-}$ mice by reconstitution with WT bone marrow confirms the relevance of CD36 expressed on corneal macrophages in TSP-1–mediated prevention of lymphangiogenesis. Consistent with these in vivo results, we noted in our in vitro experiments that exogenously added TSP-1 failed to inhibit VEGF-C expression in peritoneal macrophages with blocked or missing CD36 receptor. These results highlight an important role of monocytic cells in regulating inflammatory lymphangiogenesis in addition to hemangiogenesis (Schoppmann et al., 2002; Cursiefen et al., 2004b; Kerjaschki, 2005; Maruyama et al., 2005).

Contrary to our expectations, unlike TSP-1$^{-/-}$ macrophages, CD36$^{-/-}$ macrophages constitutively expressed significantly reduced levels of VEGF-C. This is likely caused by their reduced levels of endogenous activated TGFβ, as exogenously provided TGFβ enhanced VEGF-C expression in CD36$^{-/-}$ macrophages. This result matches with a recent study involving a chronic mouse peritonitis model (Oka et al., 2008) where the authors report absence of induction of VEGF-C, whereas their results, in fact, demonstrate significantly reduced levels of VEGF-C in the absence of endogenous TGFβ-driven signals. Therefore, a decline in constitutive VEGF-C expression in CD36$^{-/-}$ macrophages is possible because their endogenous TGFβ signaling is disrupted (Wang et al., 2009). Together, these observations imply that the inhibitory role attributed to TGFβ in lymphangiogenesis may in fact be mediated indirectly by TSP-1 bound to CD36 during activation of endogenously produced latent TGFβ. However potential signaling via TSP-1–CD36 interaction in macrophages remains largely unknown. Similar to VEGF-C, we noted inhibitory effects of TSP-1 on VEGF-D expressed by macrophages, whereas their VEGF-A expression remained unaltered.

Based on our observations, some of the complex receptor ligand interactions that lead to induction or prevention of lymphangiogenesis can be depicted as shown in Fig. S1. In WT macrophages, inhibitory signals mediated by endogenous TSP-1 negate an increased expression of VEGF-C induced by endogenous activated TGFβ resulting in no net change in VEGF-C expression. However when these WT macrophages encounter TSP-1 in their environment, the inhibitory signals via TSP-1–CD36 interactions dominate, preventing VEGF-C synthesis. In the absence of CD36, TSP-1 fails to initiate the inhibitory signal allowing TGFβ–TGFβR1 interaction to induce expression of VEGF-C, which in turn results in similar spontaneous outgrowth of corneal lymphatic vessels in the CD36$^{-/-}$ mouse (unpublished data) as shown in TSP$^{-/-}$ mice.

The cornea is one of the few avascular tissues present in the adult body, and the only one that can secondarily become vascularized by both blood and lymphatic vessels (Cursiefen et al., 2002). Whereas the corneal angiogenic privilege is redundantly organized so that absence of one or more antihemangiogenic factors does not cause spontaneous ingrowths of blood vessels (Cursiefen et al., 2004c), our present data suggest that the antilymphangiogenic privilege of the cornea is not redundantly organized. This may be related to the fact that lymphatic vessels (in contrast to blood vessels) do not compromise corneal transparency, an evolutionarily highly conserved quality (Streilein, 2003).

The identification of TSP-1 as an endogenous inhibitor of lymphangiogenesis and a factor that down-regulates VEGF-C expression opens new avenues to understand the complex interaction of angiogenesis, lymphangiogenesis, and immune response in, for example, (corneal) transplantation (Mwaikambo et al., 2006), tumorigenesis, and metastasis (Zhang and Lawler, 2007; Martin-Manso et al., 2008). In this study, we also demonstrate successful inhibition of inflammation-induced corneal lymphangiogenesis by local application of TSP-1. Thus, our work clearly presents the therapeutic implications of novel antilymphangiogenic effect of TSP-1.

MATERIALS AND METHODS

Mice. TSP-1$^{-/-}$ mice (C57BL/6 background) were originally received from the laboratory of J. Lawler (Beth Israel-Deaconess Medical Center, Harvard Medical School, Boston, MA). CD36$^{-/-}$ mice (C57BL/6 background) were obtained from the laboratory of M. Freeman (Massachusetts General Hospital, Harvard Medical School, Boston, MA). These mice were subsequently bred at the animal facility at Schepens Eye Research Institute (Boston, MA). WT control C57BL/6 mice were purchased from The Jackson Laboratory. Mice were examined using slit-lamp technology for signs of angiogenesis before euthanasia. All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmical and Vision Research. Each mouse was anesthetized by intraperitoneal injection of a mixture of 4 mg ketamine and 0.1 mg xylazine before all surgical procedures.

Induction and quantification of corneal hemangiogenesis and lymphangiogenesis. To induce corneal neovascularization, the established model of suture-induced inflammatory corneal neovascularization was applied as previously described (Cursiefen et al., 2004a,b). We have shown that suture placement into the corneal stroma in this model induces early and parallel outgrowth of both blood and lymphatic vessels into the normally avascular cornea (Cursiefen et al., 2004a,b). In brief, mice were put under general anesthesia, and 3 intrastromal 11–0 nylon sutures were placed in the corneal stroma with 2 incursions extending over 120° of corneal circumference each. This creates an inflamed hemvascularized and lymphvascularized cornea. Sutures were left in place for 7 d.

For local treatment with recombinant human TSP-1 (R&D Systems), mice received a subconjunctival injection of 50 µl TSP-1 in PBS (50 ng/50 µl) 24 h before suture placement. Just before suture placement, the corneal epithelium was scraped off to maintain good penetration of subsequently

applied TSP-1 eye drops. After suture placement, mice were treated with TSP-1 eye drops (50 ng/100 µl) three times daily until the end of the experiment and additionally received 50 ng TSP-1 via a subconjunctival injection (50 µl) on day 1 and 3 after the operation while under general anesthesia.

After 7 d, mice were euthanized and the cornea, including the limbus, was excised and flat-mount double-immunohistochemistry was performed as previously described (Cursiefen et al., 2004a,b). Corneal flat mounts were rinsed in PBS, fixed in acetone, rinsed in PBS, blocked in 2% bovine serum albumin, stained with FITC-conjugated CD31 overnight (rat α-mouse; 1:100; Santa Cruz Biotechnology, Inc.), washed, blocked, stained with LYVE-1 (rabbit α-mouse; 1:100; a lymphatic endothelium-specific hyaluronic acid receptor; gift from D.G. Jackson, Oxford University, Oxford, England, UK; Cursiefen et al., 2002, 2004a,b) washed, blocked, and stained with a Cy3-conjugated secondary antibody (goat α-rabbit; 1:100; Jackson ImmunoResearch Laboratories). The whole mounts were analyzed using an Axiophot microscope (Carl Zeiss). Digital pictures of the flat mounts were taken using Spot Image Analysis system. The area covered by LYVE-1^{+++}/$CD31^{+}$ lymphatic vessels (Fig. 1 C) was measured morphometrically on these flat mounts using the ImageJ software (National Institutes of Health). The total corneal area was outlined using the innermost vessel of the limbal arcade as the border. The total area of lymphangiogenesis was then normalized to the total corneal area, and the percentage of the cornea covered by lymphatic vessels was calculated.

Quantification of ear skin lymphangiogenesis. The analysis of ear skin for hemangiogenesis and lymphangiogenesis is described elsewhere (Cho et al., 2006; Machnik et al., 2009). We used this assay to compare the lymphatic vessel status in an angiogenic unprivileged site of TSP-$1^{-/-}$ mice against WT C57BL/6 mice.

Naive 10-d-old (p10) and 30-d-old (p30) TSP-$1^{-/-}$ and WT mice were sacrificed and their ears were harvested. Whole mounts of the ears were fixed in 5% paraformaldehyde for 24 h, washed in PBS, blocked with 3% goat serum in 0.3% Triton-X 100 in PBS, stained with rabbit anti–mouse LYVE-1 (1:200; AngioBio), washed, blocked, stained with a goat anti–rabbit Cy-3 (1:100; Dianova), washed, and analyzed using a BX51 fluorescence microscope (Olympus). Quantitative analysis were done with the Cell^F image analysis program (Olympus). The total area of lymphatic vessels was normalized to the total ear area, and the percentage of the ear section covered by lymphatic vessels was calculated.

Transmission electron microscopy. To clearly identify intrastromal isolated lymphatic vessels in TSP-$1^{-/-}$ corneas, transmission electron microscopy was performed on freshly harvested corneas of aged TSP-$1^{-/-}$ on C57BL/6 background as previously described (Cursiefen et al., 2002).

Immunohistochemistry for CD11b, CD36, CD31, and LYVE-1. Indirect immunohistochemistry was performed as previously described (Cursiefen et al., 2002, 2004a,b) on corneal whole mounts and frozen sections of normal corneas using antibodies against CD11b (rat monoclonal; BD), LYVE-1 (rabbit polyclonal; gift from D.G. Jackson), CD31 (rat monoclonal; Santa Cruz Biotechnology, Inc.), and CD36 (mouse monoclonal; Santa Cruz Biotechnology, Inc.).

Macrophage culture. Peritoneal exudate cells were collected from the peritoneal lavage of mice (age 6 wk) injected with 2% thioglycollate 3 d before. Cells were cultured in serum-free RPMI-1640 for an hour at 37°C, and nonadherent cells were discarded. Adherent cells are predominantly (>95%) F4/80^{+} macrophages as previously determined by flow cytometry. Macrophages were cultured in serum-free RPMI-1640 in the presence or absence of 5 µg/ml TSP (Hematologic Technologies, Inc.) for 24 h. In some experiments 5 ng/ml TGFβ2 (R&D Systems) or 50 µM CD36-binding peptide (CSVTCG) or control peptide (ANKHYF) were added to the culture. Peptides were synthesized by Bio-Basic Inc. Total RNA was harvested from cultured cells pooled from 3–4 wells per group and subjected to real-time PCR analysis. Three to four PCR reactions were performed per RNA sample.

Real-time PCR. Total RNA was isolated from the corneas or macrophages from WT or TSP-$1^{-/-}$ mice (8 or 24 wk; n = 4–5) using RNA STAT-60 kit (Tel-Test, Inc.) according to the manufacturer's instructions. cDNA was synthesized by reverse transcribing RNA using oligo-dT and M-MLV RT (Promega). SYBR Green real-time PCR assay (Invitrogen) was used to determine relative quantitative expression of selected genes. Sequences of the primers used for these genes are as follows: VEGF-C, forward 5′-GTTACAGAAGACCGTGTGCGAATC-3′ and reverse 5′-GATGGGACACAGCGGCATA-3′; VEGF-A, forward 5′-CTGCCGTCCGATTGAGA-3′ and reverse 5′-ACTCCAGGGCTTCATCGTTAC-3′; VEGF-D, forward 5′-ACATCAGTGCCCGAGTTAG-3′ and reverse 5′-AAGCACTTACAACCCGTATGG-3′; GAPDH, forward 5′-CGAGAATGGGAAGCTTGTCA-3′ and reverse 5′-AGACACCAGTAGACTCCACGACAT-3′. Amplification reactions were set up using SurePRIME-&GO master mix (MP Biomedicals) in triplicate with the following thermal profile: 50°C for 2 min; 1 cycle 95°C for 15 min; 1 cycle 95°C for 15 s; 52–55°C for 1 min; 72°C for 30 s; 40 cycles, 95°C for 15 s; 1 cycle on ABI Prism analyzer (Applied Biosystems). Fluorescence signal generated at each cycle was analyzed using system software. The threshold cycle (Ct) values were used to determine relative quantitation of gene expression with GAPDH as a reference gene.

VEGF-C ELISA. Culture supernatants derived from macrophages in some experiments were analyzed for the levels of VEGF-C protein using an ELISA kit (eBioscience). The antibodies used in this assay were reported to detect mouse protein (Weich et al., 2004). The assay was performed according to the manufacturer's instructions.

Flow cytometric analysis of cornea. Single-cell suspensions were prepared from corneal samples using collagenase digestion, as previously described (Saban et al., 2009). In brief, corneas were removed and minced into small fragments, followed by digestion with 2 mg/ml collagenase type IV (Sigma-Aldrich) and 0.05 mg/ml DNase I (Roche) for 1 h 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 anti-CD16/CD32 (BD) at 4°C in 0.5% BSA (Sigma-Aldrich). Subsequent antibody labeling included anti-CD11b and CD45 (BD). Staining with all antibodies was analyzed with the appropriate isotype controls.

Bone marrow transplantation. Bone marrow transplantation was performed as described previously (Cursiefen et al., 2004b; Maruyama et al., 2005). In brief, age-matched CD36$^{-/-}$ mice and WT controls (C57BL/6) were whole body irradiated, and then kept under sterile housing conditions. Within 3 d after irradiation, bone marrow transplantation was performed by injecting 10^7 cells from nonirradiated WT bone marrow into the tail vein for both irradiated CD36$^{-/-}$ and irradiated WT mice (n = 5). After 8 wk, corneal neovascularization was induced in both groups by placement of 11–0 nylon sutures intrastromally. Mice were sacrificed after 1 wk, and the degree of corneal hemangiogenesis and lymphangiogenesis quantified in CD31/LYVE-1 stained corneal flat mounts as described in Induction and quantification of corneal hemangiogenesis and lymphangiogenesis.

Statistical analysis. Statistical significance was analyzed by Mann-Whitney test. Differences were considered significant at $P < 0.05$. Each experiment was performed at least three times. Graphs were drawn using GraphPad Prism, version 3.02.

Online supplemental material. Fig. S1 shows the mechanism VEGF-C expression is regulated by CD36–TSP interaction in macrophages. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20092277/DC1.

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