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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 lymphangiogenesis. 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 monocytes, acts as an endogenous inhibitor of lymphangiogenesis.

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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 lymphatic 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 lymphatic 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
The lymphatic 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-1−/− 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 factor VEGF-C.
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 perilimbal 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. 3D). 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. 5A, 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. 5B). This finding is consistent with previous studies that were unable to demonstrate a significant expression of VEGF-C by corneal macrophages.
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.

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.
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 tissue. Our findings are further supported by the observation that 6-mo-old TSP-1−/− 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 lymphatic 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 lymphatic 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 VEGFR2 in maintaining corneal lymphatic status (Albuquerque et al., 2009) might be at work in this model.

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 monocyte 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–TGFβRI 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 anti-angiogenic 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 Ophthalmic 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 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
Bone marrow transplantation. Bone marrow transplantation was performed as described previously (Cursiefen et al., 2004b; Maruyama et al., 2005). In brief, bone marrow was harvested, and mixed 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.

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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**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’-GTTA-CAGAAAGCCGTGGCACATC-3’ and reverse 5’-GATGGGACA-CGGCCGAC-3’; VEGF-A, forward 5’-CTGGGCTGCTGGTAGAAG-3’ and reverse 5’-ACTCCAGGCTTCTGCGTTAC-3’; VEGF-D, forward 5’-ACATCGTGCCCGATTTAG-3’ and reverse 5’-AACGCTTACA-ACCCGTATGG-3’; GAPDH, forward 5’-CGAAATTGGGAGGCTTGCA-3’ and reverse 5’-AGACACCAGTACTCCAGCACAT-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 1 s, 52–58°C for 1 min; 24°C for 30 s, 40 cycles, 95°C for 1 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.


recruitment and enhances tumor cell cytotoxicity of differentiated U937 cells. Cancer Res. 68:7090–7099. doi:10.1158/0008-5472.CAN-08-0643


