Inhibition of TP signaling promotes endometriosis growth and neovascularization

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Abstract. Endometriosis is highly dependent on angiogenesis and lymphangiogenesis. Prostaglandin E_2, an arachidonic acid metabolite, has been shown to promote the formation of new blood and lymphatic vessels. However, the role of another arachidonic acid metabolite, thromboxane A_2 (TXA_2) in angiogenesis and lymphangiogenesis during endometriosis remains largely unexplored. Using a murine model of ectopic endometrial transplantation, fragments from the endometrium of WT donor mice were transplanted into the peritoneal walls of recipient WT mice (WT→WT), resulting in an increase in both the area and density of blood and lymphatic vessels. Upon transplantation of endometrial tissue from thromboxane prostanoid (TP) receptor (TXA_2 receptor)-deficient ($TP^{-/-}$) mice into $TP^{-/-}$ mice ($TP^{-/-}$→$TP^{-/-}$), an increase in implant growth, angiogenesis, and lymphangiogenesis were observed along with upregulation of pro-angiogenic and lymphangiogenic factors, including vascular endothelial growth factors (VEGFs). Similar results were obtained using a thromboxane synthase (TXS) inhibitor in WT→WT mice. Furthermore, $TP^{-/-}$→$TP^{-/-}$ mice had a higher number of $F4/80^+$ cells than that of WT→WT mice, with increased expression of genes related to the anti-inflammatory macrophage phenotype in endometrial lesions. In cultured bone marrow (BM)-derived macrophages, the levels of VEGF-A, VEGF-C, and VEGF-D decreased in a TP-dependent manner. Furthermore, TP signaling affected the polarization of cultured BM-derived macrophages to the anti-inflammatory phenotype. These findings imply that inhibition of TP signaling promotes endometrial implant growth and neovascularization.

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Abbreviations: BM, bone marrow; CCL2, C-C motif chemokine 2; CCR2, C-C motif chemokine receptor 2; COX, cyclooxygenase; IL, interleukin; i.p., intraperitoneal; LVA, lymphatic vessel area; LVD, lymphatic vessel density; LYVE-1, lymphatic vessel endothelial hyaluronan receptor 1; mPGES-1, microsomal prostaglandin E synthase-1; MR, mannose receptor; MVA, microvessel area; MVD, microvessel density; PG, prostaglandin; Prox1, prospero-related homeobox 1; TP, thromboxane prostanoid; TNF-α, tumor necrosis factor-α; TXA2, thromboxane A2; TXS, thromboxane synthase; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor

Key words: thromboxane, endometriosis, angiogenesis, lymphangiogenesis, macrophage

Introduction

Endometriosis is a serious gynecological pathology characterized by the ectopic implantation of endometrial stroma and epithelium outside the uterus (1). This disease affects ~10% of women of reproductive age and leads to numerous consequences. Despite its prevalence, patients often experience severe dysmenorrhea (painful menstruation), chronic pelvic pain, and infertility, and current therapeutic regimens frequently fail to alleviate these symptoms (2,3). Furthermore, its exact pathophysiology remains unknown.

Endometriosis is highly dependent on angiogenesis, which involves the formation of new blood vessels from pre-existing ones (4,5). In women with endometriosis, the potent pro-angiogenic stimulating factor vascular endothelial growth factor (VEGF)-A is elevated in peritoneal fluids and endometriosis lesions (4). In a model of ectopic endometriosis established by the transplantation of endometrial fragments from donor mice into the peritoneal wall of host mice, the implant area exhibited enhanced angiogenesis, as evidenced by the upregulation of pro-angiogenic factors, including VEGF-A, and endothelial cell markers, including cluster of differentiation 31 (CD31) and VEGF receptor 2 (VEGFR2), in the implant (6). Inhibition of VEGF-A suppressed the growth of endometriosis and vascular density in endometrial implants in experimental models (5,7).

Recent studies have revealed that lymphangiogenesis, the formation of new lymphatic vessels, is enhanced in endometriotic lesions (8,9). In humans, endometriosis is associated with an increased expression of pro-lymphangiogenic stimulating factors and lymphatic endothelial cell markers (4). In an ectopic endometrial transplantation model, implant growth and lymphangiogenesis were associated with increased mRNA expression of the two pro-lymphangiogenic factors *VEGF-C* and *VEGF-D* (8,9). VEGFR3, a receptor for VEGF-C and VEGF-D, kinase inhibitor reduced implant growth and lymphangiogenesis, implying that lymphangiogenesis is involved in endometriosis progression (8). However, the mechanisms underlying angiogenesis and lymphangiogenesis in endometriosis remain unclear.

Prostanoids, including prostaglandin (PG) and thromboxane (TX), are arachidonic acid metabolites. PGs have been linked to the pathogenesis of endometriosis (7,10), particularly via the cyclooxygenase-2 (COX-2)/PGE_2 pathway. COX-2 participates in the onset and progression of endometriosis (11), and enhanced expression of COX-2 has been observed in endometriotic lesions (12). In addition, VEGF-A induced by microsomal PGE synthase-1 (mPGES-1), an essential enzyme in PGE_2 synthesis, promoted endometriosis and angiogenesis of endometriotic lesions in mice (7). In terms of PGE_2-mediated lymphangiogenesis, COX-2-derived PGE_2 was found to enhance lymphangiogenesis through upregulation of pro-lymphangiogenic stimulating factors including VEGF-C and VEGF-D, in chronic inflammation (13,14), and during the healing process of gastric ulcers (15) and wounds (16).

In addition to PGE_2, TXA_2 is another representative prostanoid produced by COX and TX synthase (TXS), which exerts its activity via the TXA_2 receptor, also known as the thromboxane prostanoid (TP) receptor (17). TP plays a key role in the aggregation of platelets and in the constriction of vascular smooth muscle cells, thus contributing to cardiovascular and cerebrovascular diseases, particularly ischemic stroke, which is associated with high expression of TXA_2 (18). In mice, TP signaling facilitated blood flow recovery from hind limb ischemia via angiogenesis in ischemic tissues (19). Furthermore, TP signaling in macrophages promoted lymphangiogenesis in the diaphragm during endotoxin-induced peritonitis (20). The above findings imply that TP signaling is involved in angiogenesis and lymphangiogenesis in endometriotic lesions and promotes endometriotic growth. Thus, the present study aimed to examine the effect of inhibition of TP signaling on the development of endometriosis and the formation of new blood and lymphatic vessels.

Materials and methods

Animals. Female TP-deficient ($TP^{-/-}$) mice (8 weeks old) were generated as previously described (21). Female C57BL/6 WT mice (8 weeks old) were obtained from CLEA Japan, Inc. All experimental studies were approved by the Dean of Kitasato University School of Medicine after review by the Institutional Animal Care and Use Committee (approval no. 2022-060). The experiments were performed in accordance with the guidelines of the Science Council of Japan for animal experiments. In the present study, 75 mice were subjected to endometrial transplantation (n=39), pharmacological intervention (n=12), and cell culture (n=24).

Experimental model of endometriosis. An endometrial transplantation model was established as previously described (9). Briefly, mice were anesthetized by intraperitoneal (i.p.) injection of mixed anesthetic agents containing 4.0 mg/kg of midazolam (cat. no. 614243022; Sandoz; Novartis), 0.75 mg/kg medetomidine hydrochloride (cat. no. 14111; Nippon Zenyaku Kogyo), and 5.0 mg/kg butorphanol (cat. no. 219711; Meiji Seika Pharma). The anesthetic mixture of medetomidine, midazolam, and butorphanol has been recently used as a substitute for ketamine or pentobarbital sodium (22). The combination of anesthetics was approved by the Dean of Kitasato University School of Medicine after the review by the Institutional Animal Care and Use Committee. To rule out endogenous estrogen effects and menstrual cycle influences in mice, the bilateral ovaries were removed through paravertebral incisions. The effects of medetomidine were reversed by an i.p. injection of 0.75 mg/kg atipamezole (Nippon Zenyaku Kogyo). All donor and recipient mice were subcutaneously administered estradiol valerate (100 mg/kg) (Pelanin Depot; cat. no. 4987224136400; Mochida Seiyaku) weekly from the day of ovariectomy (6). Uterine tissues from donor mice were harvested 7 days after ovariectomy. A circular uterine fragment with a diameter of 3 mm was implanted into each side of the peritoneal wall of recipient mice and secured with 7-0 polypropylene sutures (cat. no. C0024501; B-Brown Ace Scrap, Inc.). Recipient WT or $TP^{-/-}$ mice received an implant from donor WT or $TP^{-/-}$ mice (Henceforth referred to as $WT^{-/-}\rightarrow WT^{-/-}$ and $TP^{-/-}\rightarrow TP^{-/-}$). The day of implantation was defined as day 0. Six animals received daily oral administration of ozagrel (30 mg/kg; cat. no. 87449; Kissei Pharmaceutical Co., Ltd.), a selective TXS inhibitor (20). Humane endpoints during the experiment were defined as food-water intake difficulties, weight loss ≥20% of the body weight, and reduced activities and movement.

On day 14 after endometrial transplantation, the animals were euthanized using 5% isoflurane (cat. no. 4987114133403; Pfizer) for 5 min followed by cervical dislocation, and the death was confirmed by a lack of heartbeat and respiration. Then, each side of the implanted tissue was excised. Day 0 samples were collected immediately after implantation. The excised implants were digitally photographed, and the implant area (mm^2) was determined using ImageJ version 1.53e (National Institutes of Health). The results are presented as the implant area per mm^2. One implant sample was prepared for reverse transcription-quantitative PCR (RT-qPCR), and the other for histological analysis.

Immunofluorescence analysis. Fixed endometrial samples were embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek USA, Inc.), frozen at -80˚C, and cut into 8-μm thick sections. The sections were incubated overnight at 4˚C with one of the following primary antibodies: Rabbit anti-mouse TP (1:100; cat. no. APR-069; Almone Labs, Jerusalem, Israel), rabbit anti-mouse TXS (1:100; cat. no. bs-4019R; Bioss Antibodies Inc, Woburn, MA, USA), rat anti-mouse CD31 monoclonal antibody (1:200; cat. no. 553370; BD Biosciences), rabbit anti-mouse lymphatic vessel endothelial hyaluronan receptor

Table I. Sequences of the primers used for PCR.

Gene	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
TP	CCTCCTGCTCAACACCGTTAG	CTGAACCATCATCTCCACCTC
TXS	GGATTCTGCCCAATAAGAACC	GAAGTCTCTCCGCCTCTCTTC
VEGF-A	ACGACAGAAGGAGAGCAGAAG	ATGTCCACCAGGGTCTCAATC
VEGF-C	TCTGTGTCCAGCGTAGATGAG	GTCCCCTGTCCTGGTATTGAG
VEGF-D	CCTATTGACATGCTGTGGGAT	GTGGGTTCCTGGAGGTAAGAG
CD31	CAGAGCCAGCAGTATGAGGAC	GCAACTATTAAGGTGGCGATG
VEGFR2	CTGCCTACCTCACCTGTTTCC	CGGCTCTTTCGCTTACTGTTC
VEGFR3	GGAAGGCTCTGAAGATAAAGG	ACAGAAGATGAGCAGGAGGAG
LYVE-1	GCTCTCCTCTTCTTTGGTGCT	TGACGTCATCAGCCTTCTCTT
Prox1	GTTCTTTTACACCCGCTACCC	ACTCACGGAAATTGCTGAACC
CCL2	CGGAACCAAATGAGATCAGAA	TTGTGGAAAAGGTAGTGGATG
CCR2	TTACCTCAGTTCATCCACGGC	CAAGGCTCACCATCATCGTAG
TNFα	TCTTCTCATTCCTGCTTGTGG	GATCTGAGTGTGAGGGTCTGG
IL-1β	TACATCAGCACCTCACAAGCA	CCAGCCCATACTTTAGGAAGA
IL-6	CAAAGCCAGAGTCCTTCAGAG	TAGGAGAGCATTGGAAATTGG
MR	TTTGTCCATTGCACTTTGAGG	TGCCAGGTTAAAGCAGACTTG
Fizz1	CAAGGAACTTCTTGCCAATCCAG	CCAAGATCCACAGGCAAAGCCA
IL-10	CGGAAATGATCCAGTTTTACC	TGAGGGTCTTCAGCTTCTCAC
GAPDH	ACATCAAGAAGGTGGTGAAGC	AAGGTGGAAGAGTGGGAGTTG

TP, thromboxane prostanoid; TXS, thromboxane synthase; VEGF, vascular endothelial growth factor; CD, cluster of differentiation; VEGFR, vascular endothelial growth factor receptor; LYVE-1, lymphatic vessel endothelial hyaluronan receptor 1; Prox1, prospero-related homeobox 1; CCL2, C-C motif chemokine 2; CCR2, C-C motif chemokine receptor 2; TNF-α, tumor necrosis factor-α; IL-1β, interleukin 1β; MR, mannose receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

1 (LYVE-1) (1:100; cat. no. ab14917; Abcam), goat anti-mouse LYVE-1 (1:100; cat. no. AF2125; R&D Systems Inc.), goat anti-mouse VEGF-A (1:100; cat. no. AF-493-NA; R&D Systems Inc.), rabbit anti-VEGF-C (1:100; cat. no. ab9546; Abcam), goat anti-VEGF-D (1:50; cat. no. sc6313; Santa Cruz Biotechnology, Inc.), rabbit anti-mouse CD41, a marker for platelets (1:100; cat. no. MCA2245GA; Bio-Rad Laboratories, Inc.), and rat anti-mouse F4/80, a marker for macrophages (1:100; cat. no. MCA497G; Bio-Rad Laboratories, Inc.). The sections were incubated for 1 h at room temperature with one of the following species-appropriate secondary antibodies: Alexa Fluor 488-conjugated donkey anti-rabbit IgG (cat. no. A21206), Alexa Fluor 488-conjugated donkey anti-goat IgG (cat. no. A11055), Alexa Fluor 594-conjugated donkey anti-rat IgG (cat. no. A21209) or Alexa Fluor 594-conjugated donkey anti-goat IgG (cat. no. A11058) (1:200; Molecular Probes; Thermo Fisher Scientific, Inc.). Images of stained sections were captured using a fluorescence microscope (Biozero BZ-700; Keyence Corporation). The number of $F4/80^+$ cells was counted in five fields of view (x400 magnification) of the endometrial tissue.

Determination of vessel density. Microvessel density (MVD) and lymphatic vessel density (LVD) within endometrial tissue implants have been used to assess angiogenesis and lymphangiogenesis, respectively (9). The number of $CD31^+$ blood vessels and $LYVE\text{-}1^+$ lymphatic vessels in four fields of view were counted using a fluorescence microscope (Biozero BZ-700; Keyence Corporation) (x200 magnification). The results are presented as the number of $CD31^+$ blood vessels or $LYVE\text{-}1^+$ lymphatic vessels per square millimeter (MVD/mm^2 or LVD/mm^2). In addition, the area covered by blood and lymphatic vessels was determined using ImageJ and presented as the percentage of the total area observed [microvessel area (MVA)% or lymphatic vessel area (LVA)%].

RT-qPCR analysis. Total RNA was extracted from endometriotic tissues and homogenized in TRIzol® reagent (cat. no. 15596018; Thermo Fisher Scientific, Inc.). Total RNA (1 μg) was transcribed into single-stranded cDNA using ReverTra Ace qPCR RT Kit (cat. no. FSQ-201; TOYOBO Co., Ltd.) according to the manufacturer's protocol. qPCR was performed using TB Green Premix Ex Taq II (Tli RNase H Plus; cat. no. RR820A; Takara Bio, Inc.). PCR amplification was performed under the following thermocycling conditions: Pre-denaturation at 95˚C for 10 sec, followed by a two-step PCR program consisting of 40 cycles at 95˚C for 3 sec and 60˚C for 20 sec. mRNA expression levels were calculated using the comparative threshold cycle method ($^{2\text{-}\Delta\Delta Cq}$) (23) and normalized to GAPDH expression in each sample. The sequences of the primers used are listed in Table I.

Cell preparation and culture. Bone marrow (BM) cells were isolated from the femur and tibia of 8-week-old female WT and $TP^{-/-}$ mice. BM cells were cultured in 6-well plates ($1.0x10^6$ cells/well) and maintained in RPMI-1640 medium

(cat. no. 11875-093; Gibco; Thermo Scientific) supplemented with 10% fetal calf serum and 20 ng/ml macrophage colony-stimulating factor (cat. no. 576406; BioLegend, Inc.), as previously described (9). On day 7, the cells stimulated with the TP receptor agonist U46619 (100 nM; catalog no. 16450; Cayman Chemical) were either left untreated or treated with lipopolysaccharide (10 ng/ml; cat. no. L3012; MilliporeSigma) in RPMI 1640 medium for 3 h. Cultured BM-derived macrophages were harvested and homogenized in TRIzol® reagent (cat. no. 15596018; Thermo Fisher Scientific, Inc.), and mRNA levels were determined using RT-qPCR.

Statistical analysis. All results are presented as the mean and SD. All statistical analyses were performed using GraphPad Prism version 9 (GraphPad Software, Inc.). Data between two groups were compared using an unpaired two-tailed Student's t-test, whereas comparisons between multiple groups were conducted using a one-way ANOVA followed by a Tukey's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Inhibition of TP signaling in the host enhances the development of endometriosis lesions. To investigate the involvement of TP signaling in implant growth, WT or $TP^{-/-}$ endometrial tissues were implanted into either WT or $TP^{-/-}$ recipient mice (Fig. 1). As previous data have shown that the area of endometrial implants reached a maximum on day 14 (6,9), the endometrial implant area was assessed on day 14. When $TP^{-/-}$ endometrial fragments were implanted into $TP^{-/-}$ mice ($TP^{-/-}$→$TP^{-/-}$ mice), implant growth was greater than that in WT→WT mice (Fig. 1A). Similarly, implant growth was enhanced in the WT→$TP^{-/-}$ mice; however, no statistically significant difference in implant growth was observed between $TP^{-/-}$→WT and WT→WT mice. These findings suggested that TP signaling acquired from recipients suppressed the development of transplanted endometrial fragments. Accordingly, the growth of implants as well as blood and lymphatic vessels in WT→WT and $TP^{-/-}$→$TP^{-/-}$ mice was assessed in the subsequent experiments.

On day 14, the mRNA expression levels of TXS and TP in WT→WT mouse implants increased. However, TXS mRNA expression levels in $TP^{-/-}$→$TP^{-/-}$ mouse implants did not differ from those in the WT→WT mouse implants. In addition, TP mRNA expression levels were reduced in $TP^{-/-}$→$TP^{-/-}$ mouse implants (Fig. 1B). On day 14, double immunofluorescence analysis demonstrated that TXS and TP expression in endometrial implants from WT→WT mice co-localized with those of F4/80 in stromal tissues, indicating macrophages as a source of TXS and TP in the implants (Fig. 1C).

Since TP is expressed in platelets, platelets were also assessed in endometrial tissues. Immunofluorescence analysis revealed that the distribution of CD41, a platelet marker, did not differ between WT→WT and $TP^{-/-}$→$TP^{-/-}$ mice (Fig. 1D).

Angiogenesis in endometrial implants is enhanced in $TP^{-/-}$→$TP^{-/-}$ mice. As endometrial implant growth is attributed to angiogenesis (6,9), the involvement of TP signaling in angiogenesis was investigated by counting the number of $CD31^+$ blood vessels in the WT→WT and $TP^{-/-}$→$TP^{-/-}$ mouse implants. Fig. 2A shows that the $TP^{-/-}$→$TP^{-/-}$ mouse implants displayed a greater number of newly formed $CD31^+$ blood vessels compared with WT→WT mouse implants. The MVD and MVA% in the implants of $TP^{-/-}$→$TP^{-/-}$ mice were greater than those in the WT→WT mouse implants (Fig. 2B). Additionally, mRNA expression levels of CD31, VEGFR2, and pro-angiogenic factor VEGF-A were higher in the $TP^{-/-}$→$TP^{-/-}$ mouse implants than those in the WT→WT mouse implants (Fig. 2C). Dual immunofluorescence staining demonstrated a lack of co-localization of TP with CD31 in implant tissues (Fig. 2D), suggesting that TP was not expressed in newly formed blood vessels. These findings suggest that inhibition of TP signaling does not directly facilitate angiogenesis in endometrial implants.

Lymphangiogenesis in endometrial implants is enhanced in $TP^{-/-}$→$TP^{-/-}$ mice. The role of TP signaling in lymphangiogenesis was investigated by determining LVD and LVA% (Fig. 3A and B). LVD and LVA% in $TP^{-/-}$→$TP^{-/-}$ mouse implants were higher than those in WT→WT mouse implants. The mRNA expression levels of lymphatic endothelial cell markers, including LYVE-1, VEGFR3, and Prospero-related homeobox 1 (Prox1), a marker for lymphangiogenesis (24), in $TP^{-/-}$→$TP^{-/-}$ mouse implants were higher than those in WT→WT mouse implants (Fig. 3C). The mRNA expression levels of VEGF-C and VEGF-D were also higher in $TP^{-/-}$→$TP^{-/-}$ mouse implants. These results indicate that inhibition of TP signaling stimulates lymphangiogenesis through upregulation of VEGF-C and VEGF-D. Accordingly, the localization of TP in lymphatic endothelial cells (ECs) was investigated (Fig. 3D). Dual immunofluorescence staining for TP and LYVE-1 revealed the absence of colocalization, suggesting that TP was not expressed in newly formed lymphatic vessels. Therefore, these results suggested that inhibition of TP signaling did not directly facilitate lymphangiogenesis in endometrial implants.

TXS inhibition promotes implant growth and the formation of new blood and lymphatic vessels. To confirm the role of TP signaling in endometriosis, the effects of ozagrel, a TXS inhibitor, on implant growth, angiogenesis, and lymphangiogenesis were investigated (Fig. 4). Ozagrel increased the implant area in WT→WT mice (Fig. 4A), enhanced angiogenesis and lymphangiogenesis in the implant, as evidenced by the increased MVD and MVA% (Fig. 4B), and increased LVD and LVA%, respectively (Fig. 4C). Furthermore, it enhanced the mRNA expression levels of pro-angiogenesis-related genes, including CD31, VEGFR2, and VEGF-A (Fig. 4B), and pro-lymphangiogenesis-related genes, including LYVE-1, VEGFR3, Prox1, VEGF-C, and VEGF-D in the implants (Fig. 4C).

Macrophages recruited in implants express pro-angiogenic or lymphangiogenic cytokines. Since macrophages contribute to angiogenesis and lymphangiogenesis during endometriosis development in mice (9), the number of $F4/80^+$ cells within endometrial implants was counted (Fig. 5A). The number of $F4/80^+$ cells was higher in $TP^{-/-}$→$TP^{-/-}$ mouse implants than that in WT→WT mouse implants, indicating that $F4/80^+$ cell accumulation is linked to angiogenesis and lymphangiogenesis in

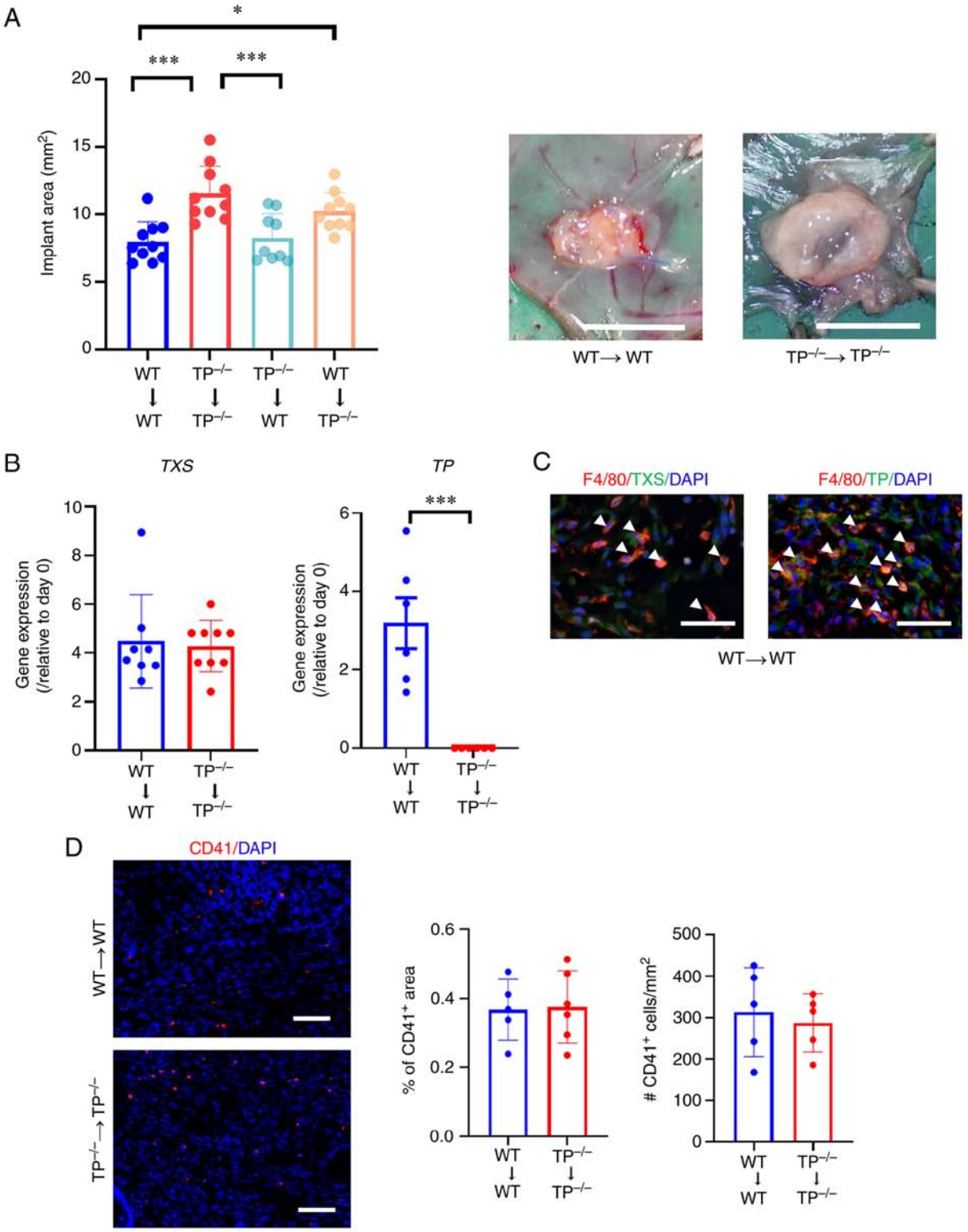

Figure 1. Endometrial implant growth and the expression levels of TXS/ TP. (A) Implant area after endometrial tissue transplantation (left panel) and typical implant appearance in WT→WT and $TP^{-/-}$→$TP^{-/-}$ mice on day 14 (right panel). Scale bar, 5 mm. (B) mRNA expression levels of *TXS* and *TP* in WT→WT and $TP^{-/-}$→$TP^{-/-}$ mouse implants. (C) Representative microphotographs of double immunostaining of F4/80 (red)/TXS (green) or TP (green) in WT→WT mouse implants on day 14. Scale bar, 50 μm. The arrowheads indicate double-positive cells. (D) Immunofluorescence staining of CD41 (red) in endometrial tissue implant sections from WT→WT and $TP^{-/-}$→$TP^{-/-}$ mice on day 14. Scale bar, 50 μm. The area and number of $CD41^+$ cells in WT→WT and $TP^{-/-}$→$TP^{-/-}$ mouse implants on day 14. Data are presented as the mean ± SD. $^*P<0.05$, $^{***}P<0.001$. TP, thromboxane prostanoid; TXS, thromboxane synthase.

the implants. In addition, enhanced $F4/80^+$ cell accumulation in implants was associated with upregulation of the macrophage chemoattractant C-C motif chemokine 2 (*CCL2*) and its receptor C-C motif chemokine 2 (*CCR2*) (Fig. 5B).

Macrophages play a pivotal role in synthesizing the VEGF protein family, which is responsible for angiogenesis (VEGF-A) and lymphangiogenesis (VEGF-C and VEGF-D). Accordingly, whether macrophages recruited in implants

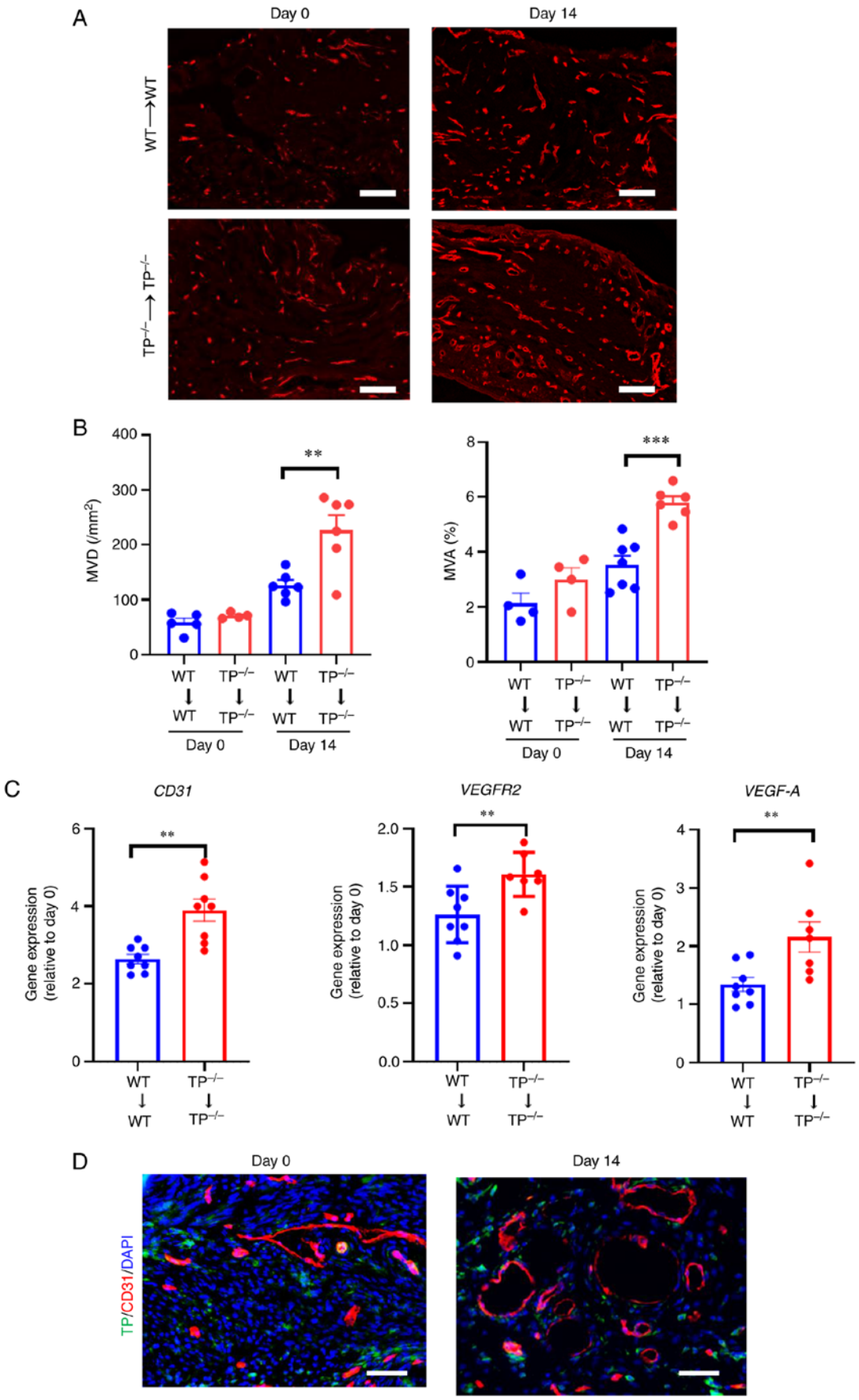

Figure 2. Inhibition of TP signaling promotes angiogenesis in endometrial implants. (A) CD31 immunofluorescence in endometrial implant sections from WT→WT and TP$^{-/-}$→TP$^{-/-}$ mice on days 0 and 14. Scale bar, 100 μm. (B) MVD and MVA in WT→WT and TP$^{-/-}$→TP$^{-/-}$ mouse implants on days 0 and 14. (C) mRNA expression levels of CD31, VEGFR2, and VEGF-A in WT→WT and TP$^{-/-}$→TP$^{-/-}$ mouse implants on day 14. (D) Double immunostaining of TP (green) and CD31 (red) in WT→WT mouse implants on days 0 and 14. Scale bar, 50 μm. Data are presented as the mean ± SD. **P<0.01, ***P<0.001. TP, thromboxane prostanoid; MVD, microvessel density; MVA, microvessel area; VEGFR, vascular endothelial growth factor receptor.

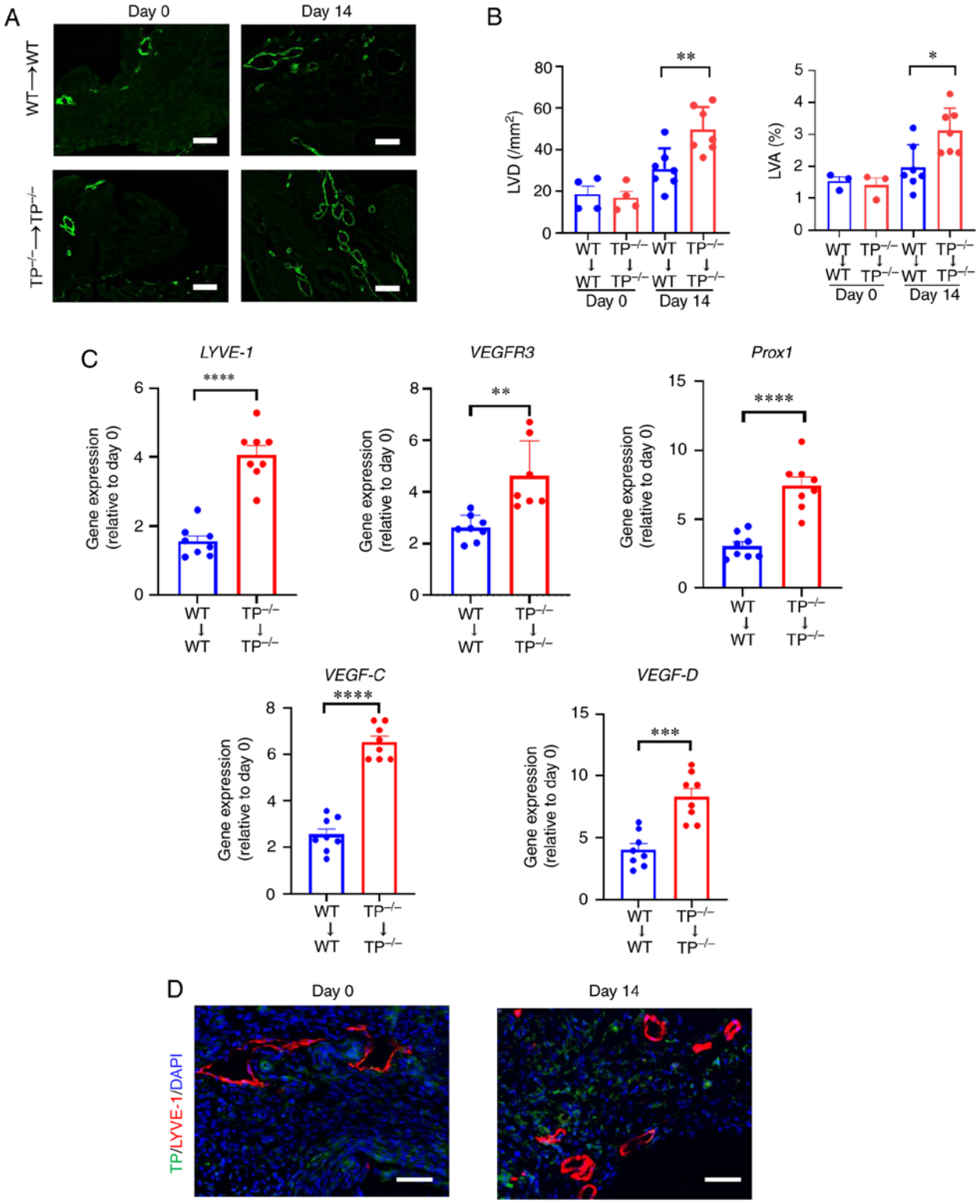

Figure 3. TP signaling inhibition promotes lymphangiogenesis in endometrial implants. (A) LYVE-1 immunofluorescence in WT→WT and TP$^{-/-}$→TP$^{-/-}$ mouse implants on day 14 Scale bar, 100 μm. (B) LVD and LVA in WT→WT and TP$^{-/-}$→TP$^{-/-}$ mouse implants on days 0 and 14. (C) mRNA expression levels of LYVE-1, VEGFR3, Prox1, VEGF-C, and VEGF-D in WT→WT and TP$^{-/-}$→TP$^{-/-}$ mouse implants on day 14. (D) Double immunostaining of TP (green) and LYVE-1 (red) in WT→WT mouse implants on day 14. Scale bar, 50 μm. Data are presented as the mean ± SD. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. TP, thromboxane prostanoid; LYVE-1, lymphatic vessel endothelial hyaluronan receptor 1; LVD, lymphatic vessel density; LVA, lymphatic vessel area; VEGFR, vascular endothelial growth factor receptor; Prox1, prospero-related homeobox 1.

expressed VEGF-A, VEGF-C, or VEGF-D was determined (Fig. 5C). Double immunofluorescence analyses demonstrated that F4/80+ cells co-localized with VEGF-A as well as VEGF-C/D expression in WT→WT mouse implants on day 14 (Fig. 5C). These findings suggest that macrophages participate in angiogenesis and lymphangiogenesis in endometrial implants through upregulation of VEGF-A and VEGF-C/D, respectively.

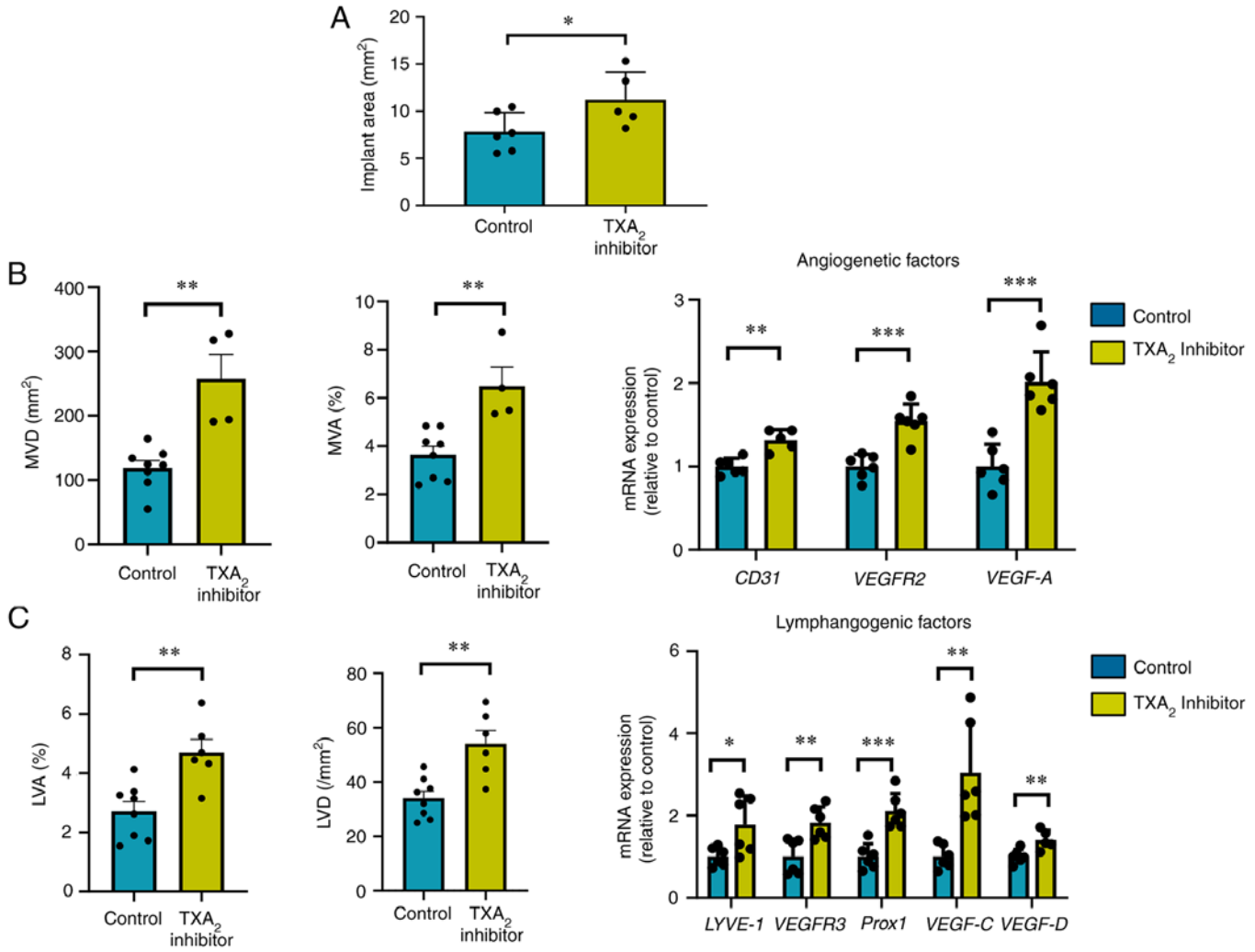

Figure 4. Effects of TXA_2 synthase inhibitor on endometrial tissue implant growth, angiogenesis, and lymphangiogenesis. (A) Implant area in TXA_2 inhibitor- or control-treated WT→WT mice. (B) MVD, MVA, and angiogenic factors in TXA_2 inhibitor- or control-treated WT→WT mouse implants. mRNA expression levels of pro-angiogenic genes CD31, VEGFR2, and VEGF-A. (C) LVD, LVA, and lymphangiogenic factors in TXA_2 inhibitor- or control-treated WT→WT mouse implants. mRNA expression levels of pro-lymphangiogenic genes LYVE-1, VEGFR3, Prox1, VEGF-C, and VEGF-D. Data are presented as the mean ± SD. *P<0.05, **P<0.01, ***P<0.001. TXS, thromboxane synthase; MVD, microvessel density; MVA, microvessel area; VEGFR, vascular endothelial growth factor receptor; LVD, lymphatic vessel density; LVA, lymphatic vessel area; LYVE-1, lymphatic vessel endothelial hyaluronan receptor 1; Prox1, prospero-related homeobox 1.

The expression levels of the anti-inflammatory factors are increased in TP-/-→TP-/- mouse implants. The mRNA expression levels of tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-6 in pro-inflammatory macrophages, and those of mannose receptor (MR), Fizz1, and IL-10 in anti-inflammatory macrophages were investigated (Fig. 6). The mRNA expression levels related to pro-inflammatory macrophage phenotypes did not differ substantially between the WT→WT and $TP^{-/-}$→$TP^{-/-}$ mice, whereas those related to anti-inflammatory macrophage phenotypes were higher in $TP^{-/-}$→$TP^{-/-}$ mice than in WT→WT mice, suggesting the role of anti-inflammatory macrophages in endometriosis growth.

TP stimulation reduces the expression of vascular growth factors and anti-inflammatory markers in cultured macrophages. Finally, whether inhibition of TP signaling in macrophages regulates pro-angiogenic or pro-lymphangiogenic factors *in vitro* was investigated. When cultured BM-macrophages were stimulated with U46619, a TXA_2 analog, the mRNA expression levels of VEGF-A, VEGF-C, and VEGF-D were downregulated in BM-macrophages from WT mice, but were not altered in those from $TP^{-/-}$ mice (Fig. 7A). These findings indicated that VEGF-A, VEGF-C, and VEGF-D levels were reduced in cultured macrophages via TP signaling.

Additionally, the mRNA expression levels of pro- and anti-inflammatory factors in macrophages were determined *in vitro*. U46619 stimulation had no effect on the expression levels of pro-inflammatory factors, including TNFα, IL-1β, and IL-6, in WT and $TP^{-/-}$ mouse macrophages. In contrast, U46619 reduced the expression levels of anti-inflammatory factors, such as MR, Fizz1, and IL-10, in WT mouse macrophages, but had no effect on $TP^{-/-}$ mouse macrophages (Fig. 7B).

Discussion

Endometriosis is associated with both angiogenesis and lymphangiogenesis. Recent studies have demonstrated that TP signaling enhances pro-angiogenic activities

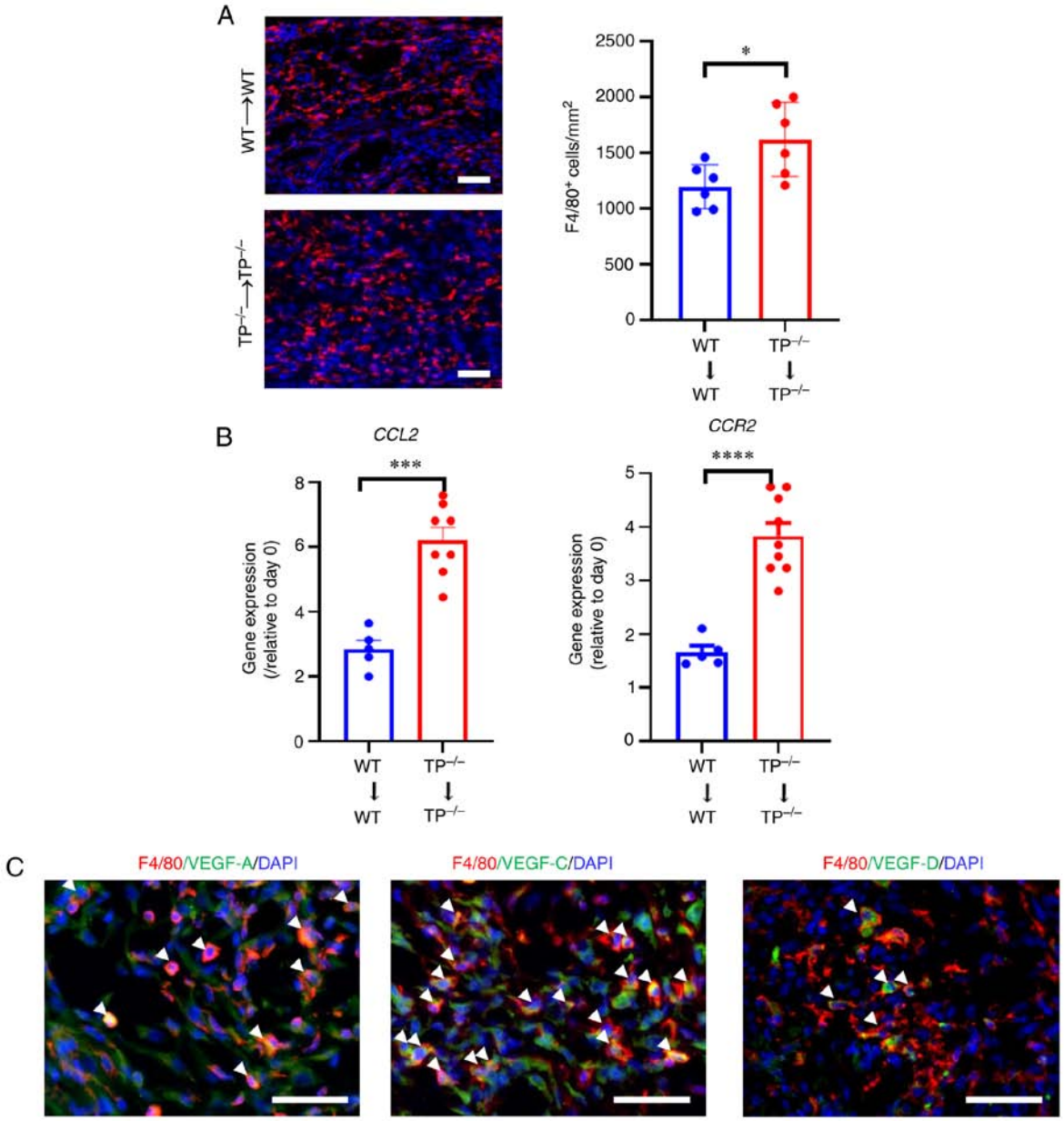

Figure 5. Macrophage accumulation in endometrial implant tissues. (A) F4/80 immunostaining (red) in WT→WT and TP$^{-/-}$→TP$^{-/-}$ mouse implants on day 14. Scale bar, 50 μm. The number of F4/80^{+} cells in WT→WT and TP$^{-/-}$→TP$^{-/-}$ mouse implants on day 14. (B) mRNA expression levels of CCL2 and CCR2 in WT→WT and TP$^{-/-}$→TP$^{-/-}$ mouse implants on day 14. (C) Double immunostaining of F4/80 (red) and VEGF-A (green), VEGF-C (green), or VEGF-D (green) in WT→WT mouse implants on day 14. Scale bar. 50 μm. The arrowheads indicate double-positive cells. Data are presented as the mean ± SD. *P<0.05, ***P<0.001, ****P<0.0001. TP, thromboxane prostanoid; CCL2, C-C motif chemokine 2; CCR2, C-C motif chemokine receptor 2; VEGF, vascular endothelial growth factor.

under pathological conditions, including inflammation and tumor growth (19,25,26). The present study was the first to demonstrate that TP signaling facilitated lymphangiogenesis in inflamed diaphragms in mice (20). Additionally, using an endometrial transplantation model, the present study revealed that inhibiting endogenous TP signaling contributed to the enhancement of angiogenesis and lymphangiogenesis in endometrial lesions in addition to endometriotic growth. TXS inhibitors increased endometrial growth and formation of new blood and lymphatic vessels. Anti-inflammatory macrophages lacking TP signaling in endometrial lesions enhanced the levels of angiogenesis and lymphangiogenesis-related cytokines. These findings suggest that activation of TP signaling mitigates endometrial growth and neovascularization.

Although the underlying mechanisms of endometriosis are unknown, angiogenesis and lymphangiogenesis in endometrial lesions contribute to its development. The pro-angiogenic factor VEGF-A is upregulated in patients and mice with endometriosis. Inhibition of VEGF-A and VEGFR1 suppresses endometriosis growth and the formation of new blood vessels in endometrial lesions (6). In addition, the pro-lymphangiogenic factors VEGF-C and VEGF-D were increased during the development of endometriosis in humans and mice (9), and VEGFR3 inhibition suppressed endometriosis growth and the formation of new lymphatic vessels in endometrial

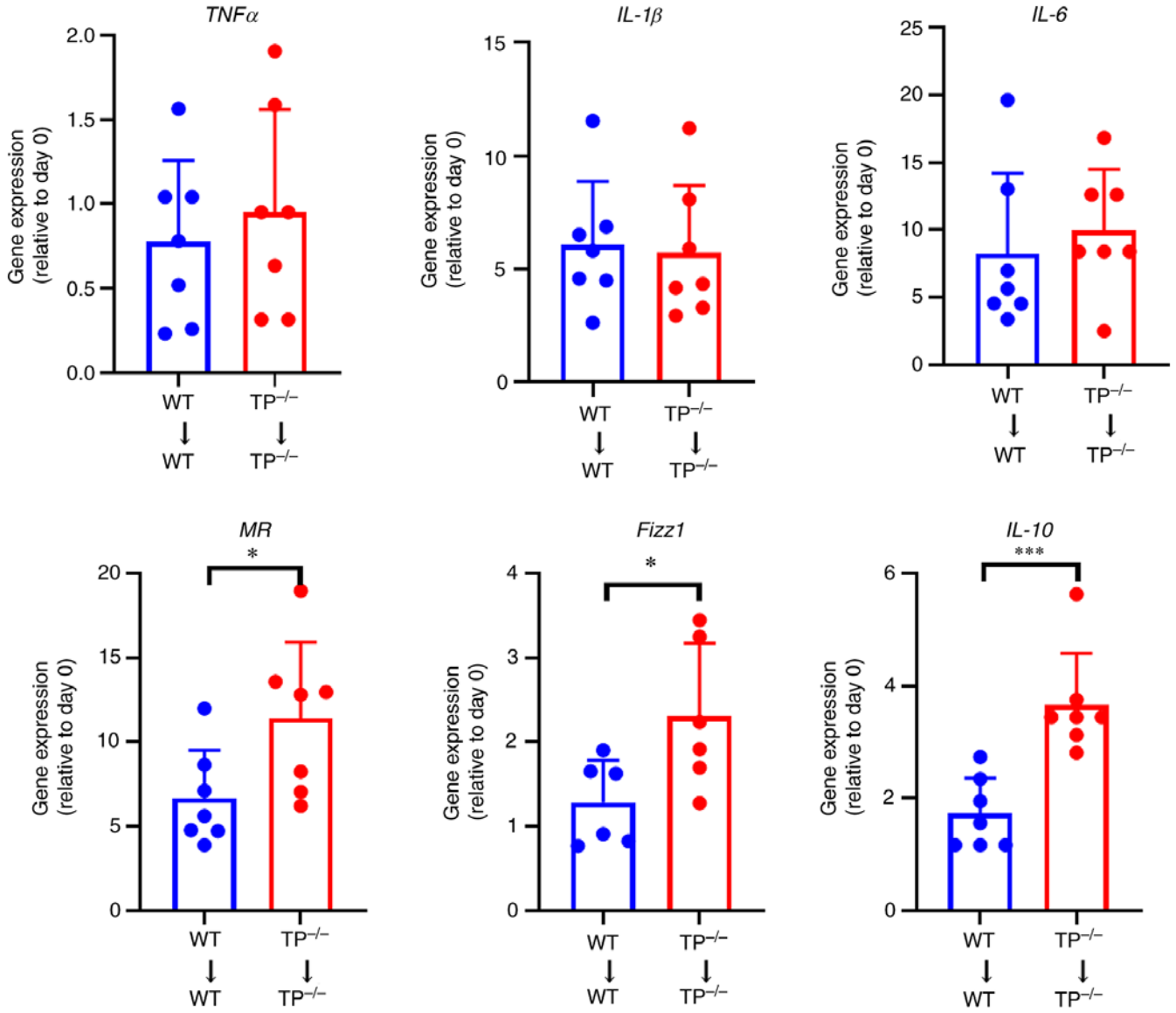

Figure 6. The mRNA expression levels of pro-inflammatory and anti-inflammatory macrophage phenotype-related genes in endometrial implant tissues. mRNA expression levels of pro-inflammatory macrophage phenotype-related genes, including TNF-α, IL-1β, and IL-6, and anti-inflammatory macrophage phenotype-related genes, including MR, Fizz1, and IL-10, in WT→WT and $TP^{-/-}$→$TP^{-/-}$ mouse implants on day 14. Data are presented as the mean ± SD. $^{*}P<0.05$, $^{***}P<0.001$. TP, thromboxane prostanoid; TNF, tumor necrosis factor; IL, interleukin; MR, mannose receptor.

lesions (8). Endometriosis was associated with overexpression of pro-angiogenic and pro-lymphangiogenic genes (27). Consistent with this, our findings revealed that the promotion of angiogenesis and lymphangiogenesis in endometrial tissue was associated with endometriotic growth.

Endometrial stromal cells have been implicated in angiogenesis and lymphangiogenesis. Macrophage accumulation in endometrial lesions was shown to be associated with neovascularization (6,8,9). BM-derived macrophages were found to be involved in endometriosis and angiogenesis (6). By contrast, large peritoneal macrophages were implicated in endometriosis (28). The accumulation of macrophages in endometrial tissues warrants further investigation. Furthermore, the specific macrophage phenotype is critical for endometriosis development (28). The results of the present study suggested that the anti-inflammatory macrophage phenotype (alternatively activated macrophages) contributed to endometriosis progression. In a murine endometriosis model, anti-inflammatory macrophages promoted the growth of endometriotic lesions, whereas pro-inflammatory macrophages protected against the disease (29). Endometriotic lesions were reduced when the anti-inflammatory macrophage phenotype expressing CD206 was deleted (30). In addition, the macrophage phenotype changes as endometriosis progresses. Pro-inflammatory macrophages appeared in the peritoneal cavity within the first 14 days after endometrial tissue implantation, and the macrophage phenotype switched to the anti-inflammatory phenotype 14 days after endometriosis induction (31). However, the oversimplification of macrophage polarization makes it difficult to understand the macrophage phenotype complex in tissues. Recent technological advances in single-cell transcript analyses have revealed five distinct subpopulations of macrophages in human endometrial lesions, suggesting the heterogeneity of macrophages during

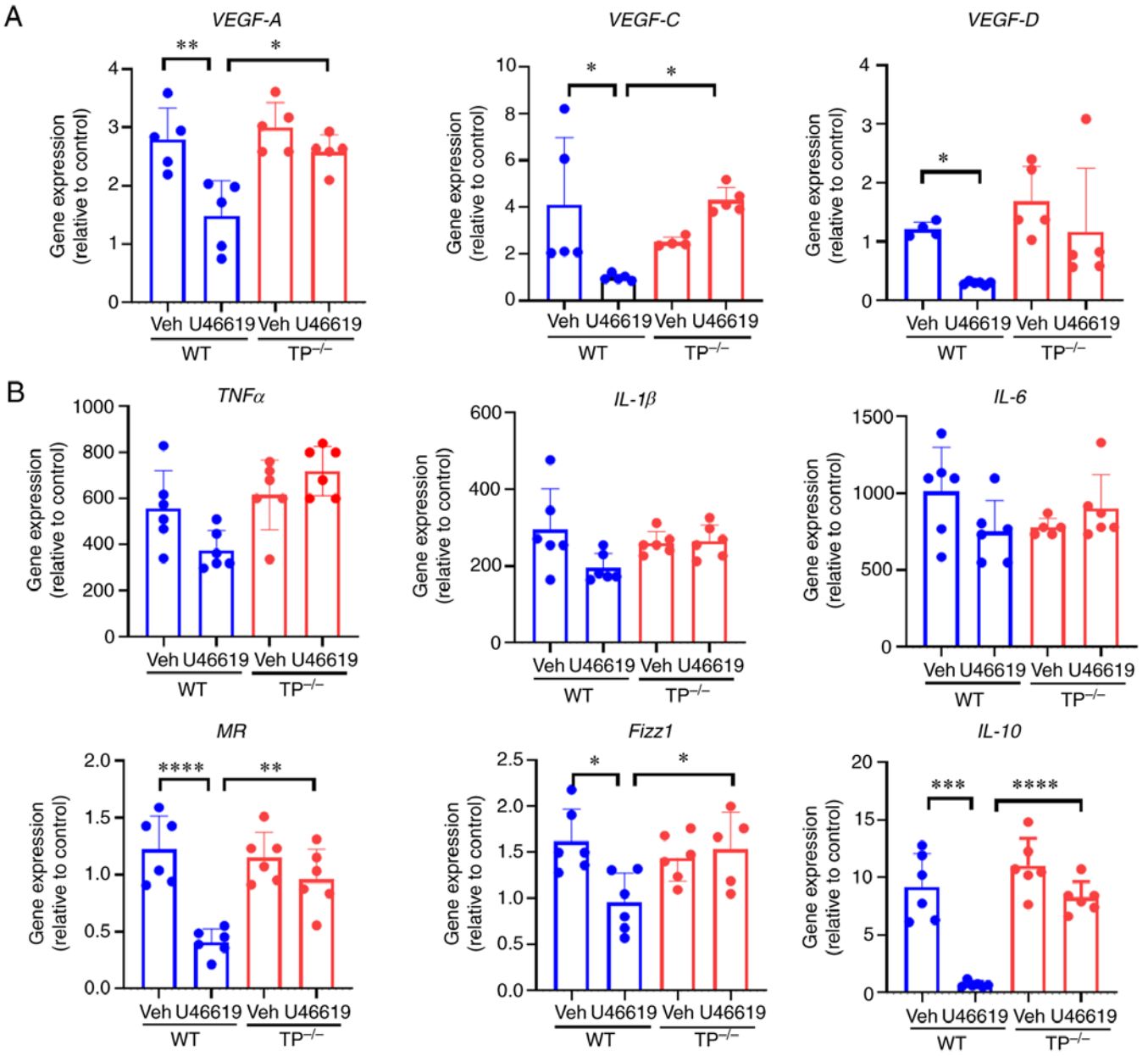

Figure 7. Vascular growth factors and pro- and anti-inflammatory macrophage phenotype-related genes in cultured macrophages. (A) Expression levels of VEGF-A, VEGF-C, and VEGF-D in cultured BM-derived macrophages stimulated with U46619 (100 nM). (B) Effects of U46619 (100 nM) on the expression levels of pro-inflammatory macrophage-related genes, including TNF-α, IL-1β, and IL-6, and anti-inflammatory macrophage-related genes, including mannose receptor (MR), Fizz1, and IL-10, in cultured BM-derived macrophages from WT and TP$^{-/-}$ mice. Data are presented as the mean ± SD. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. TP, thromboxane prostanoid; VEGF, vascular endothelial growth factor; TNF, tumor necrosis factor; IL, interleukin; MR, mannose receptor.

endometriosis development (32). Further studies are required to determine the macrophage subpopulations involved in the progression of endometriosis.

COX-2 is involved in the development of endometriosis (11). In patients with endometriosis, its expression is upregulated in the endometrial tissue (12). COX-2 inhibitors reduced endometrial growth, vascular density, and VEGF-A expression in rodent endometrial implants (7,33,34). Additionally, mPGES-1 deficient mice showed attenuated endometriosis growth and angiogenesis through the downregulation of VEGF-A in endometrial implants (7). These findings indicate that COX-2/mPGES-1-derived PGE_2 contributes to the development of endometriosis by enhancing angiogenesis. However, the role of TXA_2 in endometriosis pathology remains to be elucidated. The findings of the present study revealed that inhibition of TP signaling or TXS stimulated endometrial growth, angiogenesis, and lymphangiogenesis in endometrial lesions.

Under pathological conditions, TP signaling has both pro- and anti-angiogenic properties (35). In addition, TP signaling promotes tumor growth and angiogenesis. TXA_2 stimulates the secretion of VEGF-A from tumor cells (26). Platelet-derived TXA_2 promotes angiogenesis and blood flow recovery after hindlimb ischemia (19) and gastric ulcer (15). In contrast, TP activation of endothelial cells (ECs) reduces cell migration and proliferation (36). The activation of TP

signaling inhibits the pro-angiogenic capacity of human umbilical vein endothelial cells (HUVECs). Deletion of TP signaling in ECs enhances VEGF-induced angiogenesis *in vivo*. In addition, TP activation reduces VEGFR2 expression in HUVECs, whereas TP knockdown increases its expression (37,38). The present study showed that ablation of TP signaling enhanced angiogenesis via the production of VEGF-A from macrophages accumulated in the stroma of endometrial implants. In cultured BM-macrophages, VEGF-A expression was reduced via TP signaling. As TP was not expressed in endometrial implant microvessels, activating TP signaling did not directly induce pro-angiogenic functions in the endothelium but indirectly induced angiogenesis by releasing pro-angiogenic factors from macrophages accumulated in endometrial implants.

COX-2 inhibition prolonged inflammation-induced lymphedema in mouse tails and ears (13,14), and COX-2-derived PGE_2 was responsible for lymphangiogenesis (39). In terms of endogenous TXA_2, recent studies have shown that TP signaling in macrophages facilitated lymphangiogenesis in the diaphragm during endotoxin-induced peritonitis (20). In contrast, ablation of TP signaling enhanced lymphangiogenesis in this murine model of endometriosis, and decreased VEGF-C and VEGF-D levels via TP signaling in cultured BM-derived macrophages. TP expression was observed in endometrial stromal macrophages rather than in lymphatic vessels. These findings imply that TP signaling in macrophages suppresses lymphangiogenesis during endometriosis. However, studies on the role of TP signaling in lymphangiogenesis have yielded contradictory results, possibly because of the differences in the experimental systems used and the sex of the mice. Recruitment of pro-inflammatory macrophages to endometrial implants can also impair lymphangiogenesis, as pro-inflammatory macrophages can disrupt the formation of new lymphatic vessels at the site of injury (40).

Stimulation of macrophages with PGE_2 has been reported to change their phenotype from a pro-inflammatory to an anti-inflammatory state (16,41); however, little is known regarding the effect of TP signaling on macrophage polarization. The results of the current study suggested that ablation of TP signaling increased anti-inflammatory macrophage phenotype-related gene expression during endometriosis, including upregulation of *CCL2* expression. Increased CCL2 expression has been observed in the endometrium of women with endometriosis (42) and endometrial stromal tissues from women as well as in mice with endometriosis (43), suggesting endometrial gland and stromal cells in the implant tissues as a source of CCL2. In addition, the recruitment of anti-inflammatory macrophages was associated with the upregulation of CCL2 expression in endometrial lesions, and the CCR2 inhibitor RS102895 reduced endometrial growth in endometriotic mice (43). These data suggest that the accumulation of anti-inflammatory macrophages in endometrial lesions via the CCL2/CCR2 pathway promotes endometriosis development. Further studies are required to elucidate the role of the CCL2/CCR2 axis in the recruitment of anti-inflammatory macrophages to endometrial implant tissues from $TP^{-/-}$ mice. The application of a TP agonist (U46619) reduced anti-inflammatory macrophage phenotype-related gene expression in cultured BM-derived macrophages, suggesting that TP signaling switches the macrophage phenotype, resulting in anti-inflammatory macrophages. In contrast, a previous study found that TP stimulation increased pro-inflammatory macrophage phenotype-related gene expression in isolated peritoneal thioglycolate-induced macrophages from male mice (44). The discrepancies in the results could be attributed to differences in sex and origin of macrophages and stimulation prior to TP agonist application.

In conclusion, the results of the present study showed that inhibition of TP signaling promoted endometriosis development by enhancing neovascularization in endometrial lesions. Inhibition of TP signaling did not act on blood and lymphatic ECs, but rather on the accumulation of anti-inflammatory macrophages into the endometrial lesions. TP receptor agonist decreased anti-inflammatory macrophage phenotype-related and neovascularization-related gene expression in cultured macrophages. Therefore, the promotion of endometriosis and neovascularization are likely driven by the inhibition of TP signaling in anti-inflammatory macrophages. However, further studies are necessary to confirm this possibility. Given that selective TP receptor activation with an agonist may reduce endometrial growth, targeting TP signaling may be a viable option for treating endometriosis; however, further research is needed.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Authors' contributions

AF conceived and designed the study, performed the experiments and wrote the manuscript. KHa, ES, MT and KHo performed the experiments. MH, KS and YI performed the data analysis and interpretation, and provided technical support. MM and SN were involved in the breeding process of the $TP^{-/-}$ mice and helped to design the animal experiments. KK and HA designed the study, analyzed the data and revised the manuscript. AF, YI and HA confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The animal experiments were approved by the Animal Experimentation and Ethics Committee of Kitasato University School of Medicine (approval no. 2022-060).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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