Angiopoietin-2 promotes inflammatory lymphangiogenesis and its effect can be blocked by the specific inhibitor L1-10

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Angiopoietin (Ang)-2 promotes inflammatory lymphangiogenesis and its effect can be blocked by the specific inhibitor L1-10. Am J Physiol Heart Circ Physiol 302: H215–H223, 2012. First published November 4, 2011; doi:10.1152/ajpheart.00895.2011.— Angiopoietin (Ang)-2, a ligand of the receptor tyrosine kinase Tie2, is known to be involved in the regulation of embryonic angiogenesis. However, the role of Ang-2 in postnatal pathological lymphangiogenesis, such as inflammation, is largely unknown. We used a combination of imaging, molecular, and cellular approaches to investigate whether Ang-2 is involved in inflammatory lymphangiogenesis. We observed strong and continuous expression of Ang-2 on newly generated lymphatic vessels for 2 wk in sutured corneas of BALB/c mice. This expression was concurrent with an increased number of lymphatic vessels. TNF-α expression also increased, with peak TNF-α expression occurring before peak Ang-2 expression was reached. In vitro experiments showed that TNF-α stimulates Ang-2 and Tie2 and ICAM-1 expression on human lymphatic endothelial cells (LECs) and blood vascular endothelial cells (BECs). Ang-2 alone did not affect the biological behavior of LECs, whereas Ang-2 combined with TNF-α significantly promoted the proliferation of LECs but not BECs. In mouse models, blockade of Ang-2 with L1-10, an Ang-2-specific inhibitor, significantly inhibited lymphangiogenesis but promoted angiogenesis. These results clearly indicate that Ang-2 acts as a crucial regulator of inflammatory lymphangiogenesis by sensitizing the lymphatic vasculature to inflammatory stimuli, thereby directly promoting lymphangiogenesis. The involvement of Ang-2 in inflammatory lymphangiogenesis provides a strong rationale for the exploitation of anti-Ang-2 treatment in the prevention and treatment of tumor metastasis and transplant rejection.

inflammation; Tie2

THE LYMPHATIC VASCULAR SYSTEM, the second vascular system present in vertebrates, plays an important role in the maintenance of tissue fluid balance, immune surveillance, and the absorption of nutrients. Inflammation is the body’s normal response to injury or infection, but it may also develop during and participate in various pathological processes. In similar patterns as those observed for angiogenesis, lymphangiogenesis can be triggered by inflammation during multiple pathological processes, including transplantation (22), tumor metastasis (35), and wound healing (29). Although the importance of lymphangiogenesis in inflammatory progression is well recognized, the molecular mechanisms promoting inflammatory lymphangiogenesis are still poorly understood. Studies of the molecular regulation of lymphangiogenesis have focused mainly on the induction of inflammatory lymphangiogenesis by VEGF-C/D/A (20, 21). However, other studies have demonstrated that the expression of VEGF-C is not always upregulated during pathological lymphangiogenesis, such as in tumors with high metastatic character (1, 24), suggesting that other growth factors may be involved in postnatal lymphangiogenesis.

Angiopoietin (Ang)-2 is an important regulatory molecule in angiogenesis (14, 25, 38) and also modulates lymphangiogenesis by adjusting lymphatic specification and sprouting from the veins via Ang-2/Tie2 signaling throughout embryonic and neonatal development (33). Ang-2−/− mice display chylous ascites and defects in lymphatic vessels (14). Genetic knockin of Ang-1 into the Ang-2 locus of Ang-2-deficient mice completely rescues their lymphatic defects but not their vascular remodeling defects (14), suggesting that Ang-2 acts as an agonistic regulator in lymphatic vessels and an antagonistic regulator in blood vessels. In addition, Ang-2 plays a crucial role in postnatal remodeling and normal development and the maturation of the lymphatic vessels (14, 34). Recent studies have shown that Ang-2 is involved in inducing inflammation by sensitizing endothelial cells to TNF-α stimulation and modulating the expression of ICAMs and VCAMs (12, 39). Ang-2 also plays a role in inducing arteriogenesis by regulating smooth muscle cell coverage of neovessels (39). However, the role of Ang-2 in postnatal pathological lymphangiogenesis, such as inflammation and tumor metastasis, remains to be elucidated.

Here, we used a combination of cellular, molecular, and imaging approaches to test the possibility that Ang-2 mediates inflammatory lymphangiogenesis and found that Ang-2 sensitized lymphatic endothelial cells (LECs) to inflammatory stimuli and promoted inflammatory lymphangiogenesis. Furthermore, we demonstrated that blockade of Ang-2 can decrease inflammatory lymphangiogenesis.

MATERIALS AND METHODS

Animals. For the inflammatory neovascularization assay, we used the mouse model of suture-induced inflammatory corneal neovascularization (36). Female BALB/c mice (aged 6–8 wk) weighing 20–25 g were obtained from the animal care center of Pudong Shanghai, China. All animals involved in the study were handled in accordance with the Shanghai Jiao Tong University School of Medicine Administration Office of Laboratory Animals Guidelines for the Care and Use of Laboratory Animals and the Association for Research in Vision and Ophthalmology statement for the use of animals in ophthalmic and vision research. The investigation was approved by the Ethical Committee of Shanghai Jiao Tong University, School of Medicine, and conformed with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, Revised 1996). Before treatment, we confirmed that all mice were free from corneal diseases, using a slit lamp microscope, as well as other disorders.

Mouse suture-induced model of inflammation. Microsurgical techniques for the mouse model of sutured cornea have been previously...
described (3, 7). Mice were deeply anesthetized with an intramuscular injection of ketamine (100 mg/kg) plus xylazine (10 mg/kg) before surgical procedures and were euthanized at experimental end points by a lethal dose of CO2 asphyxiation. Three 11-0 nylon sutures (Jinhua, Shanghai, China) were placed intrastromally, with two stromal incursions extending over 120° of corneal circumference each. The outer point of suture placement was chosen near the limbus, and the inner suture point was chosen near the corneal centre equidistant from limbus, to obtain standardized angiogenic responses. Sutures were left in place for the duration of the experiment (4).

Immunofluorescence. Staining protocols were standardized for cryosections as previously described (6, 9, 11). Briefly, we used indirect immunofluorescence to localize Ang-2 in lymphatic vessels in pathologically vascularized mouse corneas as well as in normal nonvascularized mouse corneas and at the limbus. For immunofluorescence staining, murine eyes were cryopreserved in OCT embedding medium, and 5- to 7-μm cryosections were obtained. Sections were dried (15 min, 37°C) and fixed in acetone for 15 min on slides. After being rinsed with PBS (3 × 5 min), specimens were permeabilized with PBS containing 0.2% Triton X-100 for 5 min and incubated in 2% BSA in PBS at room temperature for 1 h. Specimens were incubated with the mixed primary antibody’s fluid of rabbit anti-rabbit mouse Ang-2 antibody (Abcam, 1:500) and rat anti-mouse lymphatic vessel endothelial receptor (LYVE)-1 biotin antibody (eBioscience, 1:400) in 2% BSA in PBS followed an overnight incubation at 4°C. On the second day, antibodies were rinsed with PBS (5 × 5 min) and blocked with 2% BSA for 1 h at room temperature, and specimens were then incubated with the mixed fluid of Alexa fluor 555 donkey anti-rabbit antibody (Invitrogen, Molecular Probes, 1:1,000) and streptavidin-DyLight 488 (Biolegend, San Diego, CA, 1:200) in 2% BSA in PBS for 1 h at 37°C in the dark. Next, antibodies were rinsed with PBS (3 × 15 min, on a shaker) in the dark, and specimens were incubated with 4’,6-diamidino-2-phenylindole (Invitrogen, 1:500) in PBS for 5 min at 37°C in the dark. All incubations were performed in a humid chamber. After a final rinsing step (3 × 5 min in PBS), sections were covered with DAKO (Glostrup, Denmark) fluorescent mounting medium and stored at 4°C in the dark. Fluorescence microscopy and photography were done using a confocal laser scanning microscope (Zeiss Confocal LSM 710 microscope, Carl Zeiss, Jena, Germany), and digital pictures were taken with Zen 2010 Light Edition (Soft Imaging System, Carl Zeiss).

Cells. LECs and blood vascular endothelial cells (BECs) were isolated from children’s foreskins in our laboratory as previously described (17, 18, 19). In brief, children’s foreskins were obtained by routine circumcision. After a wash and removal of the subcutaneous tissue, the epidermis was removed after dispase (2.4 U/ml, Roche, Basel, Switzerland) digestion (4°C, overnight), and the dermis was cut tissue, the epidermis was removed after dispase (2.4 U/ml, Roche, Basel, Switzerland) digestion (4°C, overnight), and the dermis was cut.

Flow cytometry analysis. LECs and BECs were seeded in six-well plates at a density of 5 × 10^5 cells/ml at 37°C for 4 h in a 5% CO2–95% O2 humid atmosphere and cultured until confluence. Cells were washed twice with PBS and then treated with different stimulation. For analysis of the expression of Ang-2 and Tie2 on LECs and BECs induced by TNF-α, LECs and BECs were exposed to vehicle or 25 ng/ml TNF-α (Cytolab, Rehovot, Israel) for 72 h, respectively. For analysis of the expression of ICAM-1 on LECs and BECs induced by TNF-α and/or Ang-2, LECs and BECs were stimulated for 72 h with or without 200 ng/ml Ang-2 (R&D Systems, Minneapolis, MN) in the presence or absence of 25 ng/ml TNF-α (Cytolab) in 100 μl/well EGM-2-MV (Clonetics) containing 1% BSA (Sigma), respectively. Subsequently, flow cytometry analysis was performed with a FACScan flow cytometry system (Becton Dickinson, San Jose, CA) as previously described (18, 26). Goat anti-human Ang-2 antibody (R&D Systems), goat anti-human Tie2 antibody (R&D Systems), and phycoerythrin (PE)-conjugated rabbit anti-goat antibody (Angiobio, San Diego, CA, USA) were used to identify Ang-2 positive cells and Tie2 expression. Phycoerythrin (PE)-conjugated mouse anti-human CD54 monoclonal antibody (BD, Bioscience, San Diego, CA) was used to identify ICAM-1 expression. Isotypic control analysis was performed in parallel. Briefly, cells were detached using 0.05% trypsin (Sigma), diluted with 4% FBS (GIBCO), filtered, and then centrifuged at 460 g for 5 min. Pellets were resuspended in 4% FBS (GIBCO) at a density of 2 × 10^5 cells/ml. For analysis of the expression of Ang-2 and Tie2, cells were then treated with vehicle, Ang-2 or Tie2 antibody, or isotype antibody at 4°C for 20 min, respectively. After centrifugation at 665 g for 5 min, pellets were resuspended in 4% FBS containing PE-conjugated antibody and incubated at 4°C for 20 min in the dark. For analysis of ICAM-1 expression, cells were treated with vehicle, PE-conjugated CD54 antibody, or isotype antibody at 4°C for 20 min, respectively. After centrifugation at 665 g for 5 min, pellet cells were resuspended once again in 4% FBS (GIBCO). Quantitative analysis was performed using FCM and Cellquest software (Becton Dickinson).

Cell proliferation assay. Cell proliferation was assessed using a [3H]thymidine (TdR) proliferation assay. LECs and BECs were cultured in 96-well plates at a density of 5 × 10^5 cells in 200 μl/well EGM-2-MV (Clonetics) at 37°C for 4 h in a 5% CO2–95% O2 humid atmosphere until adherence was formed. The medium was removed, and LECs and BECs were stimulated for 72 h with or without 200 ng/ml Ang-2 (R&D Systems) in the presence or absence of 25 ng/ml TNF-α (Cytolab) in 100 μl/well EGM-2-MV containing 1% BSA. Subsequently, cocultures were pulsed with [3H]TdR (1 μCi/well) for the final 20 h and then harvested with a Canberra Packard filter mate (Canberra Packard, Frankfurt, Germany) followed by the measurement of [3H]TdR incorporation using a Top-Count (Canberra Packard). Each experiment was analyzed in triplicate.

RNA isolation and purification. Before injury and 1, 3, 7, 11 and 14 days after suture, operated eyes were enucleated under deep anaesthesia. For each time point, total RNA was extracted from six corneas by TRIzol Reagent (Invitrogen). RNA was prepared following the manufacturer’s protocol. RNA pellets were washed with 75% ethanol, centrifuged, and dried. Pellets were dissolved in 13 μl of diethylpyrocarbonate-treated water. RNA concentration and purity were determined by measuring optical density at 260 and 280 nm using a Beckman Coulter DU 800 UV/Vis spectrophotometer (Beckman Coulter).

Real-time RT-PCR analysis. cDNA synthesis was performed using a 20-μl reaction system. cDNA was synthesized from 2 μg total RNA with thermostarch reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. Real-time RT-PCR was performed with gene-specific primers using a Stratagene Mx3000P QPCR System (Agilent Technologies, Santa Clara, CA). The sequences of primers for RT-PCR were as follows: β-actin, sense 5’-CTGTCCCTGTATGCTCTG-3’ and antisense 5’-TGTCGCGAGATTTTC-3’; Ang-2, sense 5’-TCTTCTCCAGCCCTACTAC-3’ and antisense 5’-TCTCCACCCTCTTCCATC-3’; and TNF-α, sense 5’-TCTCCATCCTTCCATC-3’ and TNF-α, sense 5’-TCTCCATCCTTCCATC-3’.
All reactions were performed in triplicate. Melting curve analyses were performed to ensure the specificity of the quantitative RT-PCRs. The data analysis was performed using the \(2^{-\Delta\Delta Ct}\) method, where \(C_t\) is threshold cycle, as previously described (23), where \(\beta\)-actin was used as the reference gene.

**Protein isolation and purification.** Before injury and 3, 5, 7, 9 and 14 days after injury, operated eyes were enucleated under deep anesthesia. For each time point, 10 corneas were placed in 400 ml of tissue protein extraction reagent (Thermo Scientific, Pierce Biotechnology) supplemented with a protease inhibitor cocktail (Merck, Darmstadt, Germany) followed by homogenization with a hand-held and power-driven drill. After corneas were homogenized, the suspension was incubated on ice for 10 min to allow lysis. Lysates were cleared of debris by centrifugation.

After corneas were homogenized, the suspension was incubated on ice for 2 ha t room temperature followed by an incubation with rabbit anti-mouse Ang-2 antibody (Abcam) and goat anti-mouse \(\beta\)-actin polyclonal antibody (Abcam). After an extensive wash with 0.1% Tween 20 in PBS, membranes were incubated for 1 h at 37°C in the dark with IRDye 800DX-conjugated affinity-purified donkey anti-rabbit antibody (Rockland Immunochemicals) and IRDye 700DX-conjugated affinity-purified donkey anti-goat antibody (Rockland). After a further wash, luminescence signals were measured using the LI-COR Odyssey infrared imaging system and corresponding software.

**Suture-induced inflammatory corneal neovascularization assay.** Mouse models of suture-induced corneal neovascularization were randomly divided into two groups. The treatment group (n = 12) received L1-10 (4 mg/kg), a FC-fusion protein Ang-2-specific inhibitor (kindly provided by Amgen), dissolved in PBS, injected subcutaneously every other day starting with 1 day before surgery. The control group (n = 12) received an equal amount of PBS solution. Mice were killed after 7 or 14 days. Corneal hemangiogenesis and lymphangiogenesis were determined via whole mount immunofluorescence staining.

**Whole mount preparations and immunofluorescence staining.** Preparations were done as previously described (6). In brief, mice were killed under deep anesthesia, the operated eyes were enucleated, and the corneas were dissected from the eyes behind the corneal limbus. Corneas were washed in PBS (3 × 5 min) at room temperature. Fixation was done by acetone for 30 min. After three additional wash steps in PBS and blockade with 2% BSA plus 0.3% Triton X-100 in PBS for 2 h at room temperature, corneas were stained overnight at 4°C with rabbit anti-mouse LYVE-1 antibody (Abcam, 1:500) with 2% BSA in PBS. On the second day, after a wash in PBS (5 × 5 min), the antibody was blocked with 2% BSA in PBS for 2 h. The secondary antibody, Alexa fluor 488 rat anti-mouse CD31 (Biolegend, 1:50), with 2% BSA in PBS, was added for an overnight incubation at 4°C in the dark. On the third day, after a wash in PBS (5 × 5 min), the antibody was blocked with 2% BSA in PBS for 2 h. The third antibody, Alexa fluor 555 donkey anti-rabbit antibody (Invitrogen, 1:1,000), with 2% BSA in PBS, was incubated for 45 min at room temperature in the dark. As a final step, antibody was washed in PBS (3 × 15 min). Corneas were moved to microscope slides, covered with DAKO fluorescent mounting medium, and stored at 4°C in the dark. Fluorescence microscopy and photography were done using a confocal laser scanning microscope (Zeiss Confocal LSM 710 microscope, Carl Zeiss), and digital pictures were taken with Zen 2010 Light Edition (Soft Imaging System, Carl Zeiss).

**Dynamic functional and statistical analyses and graphs.** Quantitative analysis of blood and lymphatic vessels was performed in a standardized procedure using Image-pro plus 6.0 (Soft Imaging Sys-

![Fig. 1. Representative images of angiopoietin (Ang)-2 in immunofluorescence-stained corneas.](http://ajpheart.physiology.org/)
tem) software by means of threshold analysis. For measurements, we used rectangles of a standardized size (1.1 mm²), which were aligned along the limbus as previously described (3). The corneal area filled with blood or lymphatic vessels (hemvascularized or lymphvascularized area) was measured in each rectangle. Vessel area ratio was determined by the vascularized area of the treatment group in relation to that of the control group. The vascularized areas of the control groups were defined as being 100%. Analysis of differences of the two samples was carried out using a standard Student two-tailed t-test (SPSS 17.0 statistical software, SPSS, Chicago, IL). Values are presented as means ± SE. P values of <0.05 were considered as significant. In the figures, asterisks or daggers are used to illustrate a significant difference between groups (P < 0.05) and two asterisks are used to illustrate a significant difference between groups (P < 0.01). Graphs were drawn using origin7.5 (Originlab).

RESULTS

Expression of Ang-2 on physiological lymphatic vessels. Untreated murine eyes were used to investigate whether physiological lymphatic vessels at the limbus (junction of the vascularized conjunctiva and nonvascularized cornea) ex-

Fig. 2. Expression of Ang-2, Tie2, and ICAM-1 on LECs and blood vascular endothelial cells (BECs) and the proliferation assay of LECs and BECs in vitro. A: after the stimulation of TNF-α, Ang-2-positive cells of LECs and BECs were obviously increased compared with the control, as evidenced by flow cytometry analysis. B: Tie2 expression on LECs and BECs was markedly upregulated after stimulation of TNF-α compared with the control, as evidenced by flow cytometry analysis. C: LECs and BECs appeared to have low-level expression of ICAM-1 before stimulation (1 and 5). Ang-2 had no obvious effect on ICAM-1 expression on LECs and BECs (2 and 6), whereas ICAM-1 expression on LECs and BECs was obviously enhanced under the stimulation of TNF-α (3 and 7). ICAM-1 expression on LECs and BECs stimulated by the combination of Ang-2 and TNF-α was stronger than that stimulated by TNF-α alone (4 and 8). D: proliferation of LECs and BECs was assessed using a [³H]Thymidine (Tdr) proliferation assay. Ang-2 did not stimulate the proliferation of LECs and BECs by 10.220.32.246 on October 15, 2017 http://ajpheart.physiology.org/ Downloaded from
pressed Ang-2 by immunofluorescence staining. We found that no lymphatic vessels were found in the cornea except in the limbus (Fig. 1A), and a low-level expression of Ang-2 colocalized on the resting LYVE-1-positive lymphatic vessels at the limbal arcades and adjacent physiological vascularized conjunctiva in the normal, nontreated cornea (Fig. 1D). However, some inside epithelial cells near the matrix also expressed Ang-2 (Fig. 1B).

Expression of Ang-2 on inflammatory lymphatic vessels. In pathologically vascularized murine corneas, we localized Ang-2 in corneas by immunofluorescence staining and observed that inflammation obviously induced lymphangiogenesis (Fig. 1E) and that Ang-2 expression was simultaneously strongly upregulated (Fig. 1F) in the sutured cornea. We also found that Ang-2 was expressed strongly by the newly generated lymphatic vessels (Fig. 1H).

**TNF-α increases the expression of Ang-2 and Tie2 on LECs and BECs.** TNF-α is regarded as a key inflammatory cytokine and plays a major role in the initiation of inflammation (5, 32). To assess the effect of TNF-α on the expression of Ang-2 and Tie2, LECs and BECs were harvested from children’s foreskins and cultured with or without TNF-α for 72 h. Flow cytometry analysis confirmed that stimulation of TNF-α led to enhanced expression of Ang-2 and Tie2 on both LECs and BECs (Fig. 2, A and B), indicating that inflammation triggers the Ang-2/Tie2 signaling pathway of endothelial cells by upregulating Ang-2 mRNA expression, releasing more Ang-2 and enhancing expression of its receptor, Tie2.

**Ang-2 regulates ICAM-1 expression on LECs and BECs under an inflammatory setting.** ICAM-1 is known to play an important role in inflammation by mediating the adherence of leukocytes to the endothelium and initiating extravasation of these cells (2, 37). Ang-2 regulates TNF-α-induced ICAM-1 expression on vascular endothelial cells and has a crucial role in the induction of inflammation (12). A previous study (10) has also shown that ICAM-1 induces angiogenesis and supports the survival of microvessels. However, the role of ICAM-1 in inflammatory lymphangiogenesis is poorly understood. Here, we analyzed ICAM-1 expression on LECs and BECs upon stimulation with or without Ang-2 in the presence or absence of TNF-α. Ang-2 alone had no effect on ICAM-1 expression on LECs and BECs (Fig. 2, C and D), whereas ICAM-1 expression on LECs and BECs was enhanced by stimulation with TNF-α (Fig. 2, C and D). Interestingly, ICAM-1 expression on LECs and BECs stimulated by the combination of Ang-2 and TNF-α was stronger than that of Ang-2 or TNF-α alone, as assessed by flow cytometry analysis (Fig. 2, C and D).

Ang-2 regulates the proliferation of LECs in the presence of TNF-α. Since Ang-2 is regarded as a key player in developing LECs (14), we then used cell proliferation assays to assess the effect of Ang-2 on LECs in an inflammatory setting. LECs and BECs were cultured with or without Ang-2 in the presence or absence of TNF-α for 72 h. Ang-2 alone did not stimulate the proliferation of LECs in the absence of TNF-α, and it inhibited BEC proliferation. TNF-α alone stimulated the proliferation of BECs in the absence of Ang-2 but inhibited the proliferation of LECs. Ang-2 combined with TNF-α significantly stimulated the proliferation of LECs, whereas the proliferation of BECs was not enhanced by the same treatment (Fig. 2D).

Expression of Ang-2 and TNF-α is upregulated in sutured corneas. To further explore the correlation between Ang-2 and TNF-α in inflammatory lymphangiogenesis, we used a mouse model of suture-induced corneal neovascularization. Quantitative RT-PCR analysis was used to evaluate the level of Ang-2 and TNF-α expression. Both Ang-2 mRNA and TNF-α mRNA were induced in inflamed corneas (Fig. 3A). TNF-α mRNA expression increased dramatically soon after suture, peaked on the seventh day, and then declined (Fig. 3A). Along with inflammatory lymphangiogenesis in sutured corneas, Ang-2 mRNA expression increased for at least 14 days, and its peak lagged behind that of TNF-α mRNA expression (Fig. 3A; see also Fig. 5A). The observation of dynamic Ang-2 expression was further assessed with Western blot analysis, which showed that Ang-2 expression was gradually upregulated for ~2 wk (Fig. 3B). The number and density of inflammatory lymphatic vessels and blood vessels increased during this time (Figs. 4F and 5A).
Systemic application of L1-10 inhibits lymphangiogenesis but increases hemangiogenesis. Whole mount staining showed that blood and lymphatic vessels were physiologically present at the limbal border between the cornea and conjunctiva in our study (Fig. 4, A and B), and normal mouse corneas were devoid of lymphatic and blood vessels (Fig. 4, A and B); however, 2 wk after suture, corneal lymphatic and blood vessels were scattered through the stroma (Fig. 5A). To determine whether Ang-2 acts as an agonistic regulator of inflammatory lymphangiogenesis, we used L1-10, a specific inhibitor of Ang-2, in mouse models. L1-10 was injected into mice subcutaneously every other day starting at 1 day before surgery. The treatment group received L1-10 (4 mg/kg) dissolved in PBS, whereas the control group received an equal amount of PBS. Mice were treated for 7 or 14 days after suture. After 7 days, the treatment group (n = 5) showed significantly reduced lymphatic growth (P < 0.01; Fig. 4J) and increased blood vessel growth with increased vascular density but thinner in diameter (P < 0.05; Fig. 4K) compared with the control group (n = 5). When sutures were left in for 2 wk, the treatment group (n = 7) again displayed a significant decrease in the outgrowth of lymphatic vessels (P < 0.01; Fig. 5C) and an increase in hemangiogenesis (P < 0.01; Fig. 5D) compared with the control group (n = 7). Thus, blockade of the Ang-2/Tie2 system by L1-10 efficiently inhibited inflammatory lymphangiogenesis, suggesting that Ang-2 is a key trigger of inflammatory lymphangiogenesis.

DISCUSSION

Our results demonstrate that Ang-2 is only weakly expressed on the resting lymphatic vessel endothelium but that inflammation induces Ang-2 expression and lymphangiogenesis. Up-regulation of Ang-2 expression on newly generated inflammatory lymphatics occurred simultaneously with inflammatory lymphangiogenesis. These observations clearly demonstrate...
that Ang-2 expression is tightly controlled and dynamic and that it is induced and dramatically upregulated by exogenous stimuli that trigger the process of lymphatic growth, such as inflammation. To our knowledge, this is the first report of Ang-2 expression on inflammatory lymphatic vessels suggesting that Ang-2 may play a major role in inflammatory lymphangiogenesis.

We further observed that Ang-2 does not directly affect LECs but rather appears to prime quiescent LECs and control their responsiveness to pro-inflammatory cytokines. LECs underwent a transition from a resting or inhibiting state to an active state when treated with a combination of Ang-2 and TNF-α. Activation of LECs, in turn, resulted in increased autocrine expression of Ang-2, Tie2, and adhesion molecules and the proliferation of the cell line, suggesting that this process might contribute to lymphatic neovascularization. It is interesting to note that Ang-2 significantly induced the proliferation of LECs but not BECs in an inflammatory setting, despite the fact that inflammation promoted the expression of Ang-2, Tie2, and ICAM-1 in both endothelial cell lines. This indicates that the different vasculature systems are differentially susceptible to exogenous stimuli and/or that the mechanisms of lymphangiogenesis and blood vascular angiogenesis are distinct.

We observed a strong induction of both Ang-2 mRNA and TNF-α mRNA in inflamed corneas, with the peak of TNF-α expression after injury being reached before that of Ang-2 expression. Ang-2 expression was gradually upregulated for ~2 wk after injury, at both the mRNA and protein levels, and the number and density of inflammatory lymphatic vessels and blood vessels also increased during this time. We therefore hypothesize that, under inflammatory conditions, pro-inflammatory cytokines such as TNF-α induce and upregulate Ang-2 expression on LECs. As a result, Ang-2 can be rapidly released from endothelial Weibel-Palade bodies in response to stimuli, such as inflammation and hypoxia (13, 27, 41), thereby promoting inflammatory lymphangiogenesis. It appears that Ang-2 cooperates with TNF-α to initiate lymphangiogenesis during an early stage of inflammation after injury (about the first week in mice) and that the expression of TNF-α then declines. The proliferation and survival of LECs appear to mainly depend on the stimulation of Ang-2, possibly through an internal autocrine loop mechanism (31).

To elucidate the direct contribution of Ang-2 to inflammatory lymphangiogenesis, we treated sutured corneas in mice with the FC-fusion protein L1-10, which inhibits Ang-2 binding to its receptor Tie2 and suppresses the proliferation of endothelial cells (28). Recent studies have shown that L1-10 interferes with Ang-2-modulated angiogenesis, vascular patterning, remodeling, and other functions (39, 40) and that L1-10 inhibits the expression of ICAM and VCAM in ischemic tissue (39), suggesting that L1-10 is a specific and strong inhibitor of Ang-2. One of the most intriguing findings of our study is that inhibition of the Ang-2/Tie2 system using L1-10 efficiently blocked inflammatory lymphangiogenesis. These functional data add weight to the suggestion that Ang-2 is a key trigger of inflammatory lymphangiogenesis. A recent study (15) showed that lymphangiogenesis and inflammation are significantly reduced in an inflammatory bowel disease model using Ang-2−/− mice, supporting the hypothesis that Ang-2 is one of the key regulators involved in inflammatory lymphangiogenesis. Previous studies have shown that inflammatory...
lymphangiogenesis can be inhibited by blocking several signaling pathways, including the VEGF-C/D/VEGF receptor (VEGFR)-3 signaling pathway (16, 30) and the VEGF-A/VEGFR-2 signaling pathway (3). Here, we show, for the first time, that inflammatory lymphangiogenesis can also be blocked by the Ang-2 inhibitor L1-10, which provides a novel rationale for the inhibition of postnatal pathological lymphangiogenesis. Conversely, these results further prove that the Ang-2/Tie2 signaling pathway is one of the most important signaling pathways regulating postnatal lymphangiogenesis, although direct evidence for a relationship between the Ang-2/Tie2 and VEGF-C/D/VEGFR-3 or VEGF-A/VEGFR-2 signaling systems in inflammatory lymphangiogenesis is lacking. Furthermore, we found that blockade of the Ang-2/Tie2 system stimulated inflammatory blood vessel growth.

Collectively, our data demonstrate that Ang-2 acts as an agonistic regulator in inflammatory lymphangiogenesis and an antagonistic regulator in inflammatory angiogenesis and that the Ang-2/Tie2 signaling pathway is an important system in the regulation of pathological lymphangiogenesis. Our work also provides compelling evidence that the molecular mechanisms controlling inflammatory lymphangiogenesis and angiogenesis are very different. Ang-2 sensitizes the blood vasculature to response inflammatory stimuli but does not promote angiogenesis. In contrast, Ang-2 not only sensitizes the lymphatic vasculature during inflammation but also plays a crucial role in promoting lymphangiogenesis. Elucidating the role of Ang-2 in inflammatory lymphangiogenesis might further our understanding of multiple pathological processes, and the finding that Ang-2 is involved in the control of inflammatory lymphangiogenesis suggests that anti-Ang-2 treatment might be useful in the prevention and treatment of tumor metastasis and transplant rejection.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: Z.-X.Y. and Z.-H.J. performed experiments; Z.-X.Y., M.-F.L. drafted manuscript; N.-F.L., conception and design of research; N.-F.L., edited and revised manuscript; N.-F.L., approved final version of manuscript.

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