IL-37 Is a Novel Proangiogenic Factor of Developmental and Pathological Angiogenesis

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Angiogenesis, the formation of new blood vessels from pre-existing vasculature, is tightly controlled by proangiogenic and antiangiogenic cytokines.1 Those cytokines act on endothelial cells (ECs), which migrate, proliferate, and give rise to new vascular bed.2 Inappropriate angiogenic signaling results in disordered neovascularization, which contributes to diseases, such as ocular neovascularization, rheumatoid arthritis and atherosclerosis. Strategies modulating the prominent angiogenic cytokines such as vascular endothelial growth factor (VEGF) have shown therapeutic efficacy.3 However, a proportion of patients are refractory to VEGF blockade therapy, suggesting other factors that coordinately contribute to angiogenesis remain to be identified.4

Many cytokines of the interleukin-1 (IL-1) family, such as IL-1α, IL-1β, IL-18, and IL-33, contribute importantly to inflammatory responses and angiogenesis.5–8 IL-37, a recently identified member of the IL-1 family that until recently had no murine homolog, was suggested to function as a fundamental anti-inflammatory cytokine. IL-37 is produced as a precursor protein that is processed by caspase-1, releasing the mature form of IL-37.9 As an intracellular cytokine, IL-37 translocates to the nucleus and interacts with Smad3 to exert inflammatory function.10 IL-37 was recently suggested to bind to IL-18Rα and IL-1R8, which function together to mediate the inflammatory activity of IL-37.11 IL-37 is detected in many tissues, including lymph nodes, thymus, and bone marrow, as well as in monocytes, epithelial cells, and breast carcinoma cells.12,13 IL-37 is upregulated in the serum of patients with systemic lupus erythematosus, Guillain–Barré syndrome, hepatitis B virus infection, and amyotrophic lateral sclerosis,14–17 but the function of IL-37 as a secreted cytokine remains unclear. Notably, IL-37 is probably involved in angiogenesis-associated diseases, inflammation, and sterile inflammatory conditions, such as tissue injury, B virus infection, and amyotrophic lateral sclerosis.18,19 IL-37 is thought to function as a secreted cytokine to exert anti-inflammatory effects, but the function of IL-37 as a secreted cytokine remains unclear. Notably, IL-37 is probably involved in angiogenesis-associated diseases and inflammatory conditions.

Objective—Angiogenesis is tightly controlled by growth factors and cytokines in pathophysiological settings. Interleukin 37 (IL-37) is a newly identified cytokine of the IL-1 family, some members of which are important in inflammation and angiogenesis. However, the function of IL-37 in angiogenesis remains unknown. We aimed to explore the regulatory role of IL-37 in pathological and physiological angiogenesis.

Approach and Results—We found that IL-37 was expressed and secreted in endothelial cells and upregulated under hypoxic conditions. IL-37 enhanced endothelial cell proliferation, capillary formation, migration, and vessel sprouting from aortic rings with potency comparable with that of vascular endothelial growth factor. IL-37 activates survival signals including extracellular signal-regulated kinase 1/2 and AKT in endothelial cells. IL-37 promoted vessel growth in implanted Matrigel plug in vivo in a dose-dependent manner with potency comparable with that of basic fibroblast growth factor. In the mouse model of retinal vascular development, neonatal mice administrated with IL-37 displayed increased neovascularization. We demonstrated further that IL-37 promoted pathological angiogenesis in the mouse model of oxygen-induced retinopathy.

Conclusions—Our findings suggest that IL-37 is a novel and potent proangiogenic cytokine with essential role in pathophysiologic settings. (Arterioscler Thromb Vasc Biol. 2015;35:00-00. DOI: 10.1161/ATVBAHA.115.306543.)

Key Words: cytokines ■ endothelial cell ■ inflammation ■ interleukins ■ oxygen

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pathologies. For example, IL-37 is detected in tumor cells of breast carcinoma and infiltrating plasma cells in colon carcinoma. Moreover, IL-37 is upregulated in the synovial tissue from patients with rheumatoid arthritis. However, to the best of our knowledge, no studies have linked IL-37 to the function of ECs.

In this study, we have found that IL-37 is a novel and potent proangiogenic cytokine in developmental and pathological angiogenesis. We provide evidence that IL-37 enhanced EC proliferation, migration, and capillary formation in vitro and promoted vessel sprouting from aortic rings ex vivo with potency comparable with that of VEGF. Matrigel plugs incorporated with increasing dose of IL-37 displayed increased angiogenesis in a dose-dependent manner. Furthermore, we have used models of developmental and pathological angiogenesis to show that IL-37 promoted neovascularization in mouse model of retinal vascularization and oxygen-induced retinopathy (OIR). These results unveiled IL-37 as a novel regulator of pathophysiological angiogenesis.

Materials and Methods

Materials and methods are available in the online-only Data Supplement.

Results

IL-37 Expression Is Upregulated by Hypoxia in ECs

Currently, no studies to our knowledge have demonstrated endothelial expression of IL-37. We thus determined the expression of IL-37 in ECs and found that IL-37 protein expression were detected in both large and small vascular ECs, including human umbilical vein endothelial cells (HUVECs) and human microvascular endothelial cells (HMEC-1; Figure 1A in the online-only Data Supplement). As hypoxia is an important pathophysiological condition for the induction of angiogenesis, to determine whether IL-37 expression is regulated by hypoxia, HUVECs were cultivated in hypoxic environment (0.1% O2) for 24 hours. The expression of IL-37 was induced by hypoxia as determined by real-time polymerase chain reaction (Figure 1B in the online-only Data Supplement). Because IL-37 is produced as a precursor that needs to be processed to release its mature form, we analyzed both pro- and mature form of IL-37 in HUVECs under hypoxic conditions. Both pro- and mature form of IL-37 were markedly induced by hypoxia in HUVECs (Figure 1A), suggesting that hypoxia induced expression and maturation of IL-37. We thus further determined whether IL-37 secretion can be induced by hypoxia. As shown in Figure 1B, hypoxia stimulated IL-37 secretion in the supernatant of HUVECs as determined by ELISA.

Moreover, to understand the mechanism underlying the upregulation of IL-37 during hypoxia, we have knocked down HIF-1α, the principle transcriptional activator that orchestrates hypoxia response, and examined the expression levels of IL-37. The result showed that knockdown of HIF-1α expression inhibited hypoxia-induced upregulation of IL-37 mRNA and protein levels, indicating that IL-37 expression was regulated by proangiogenic hypoxic signaling (Figure II in the online-only Data Supplement). Consistently, we observed that IL-37 expression

Figure 1. Interleukin (IL)-37 expression and secretion in human endothelial cells. A, IL-37 expression was upregulated in human umbilical vein endothelial cells (HUVECs) after incubation under hypoxic conditions (0.1% O2). Pro- and mature IL-37 are indicated by arrows. B, Quantification of IL-37 in the supernatants of HUVECs incubated under hypoxic conditions as determined by ELISA. C, IL-37 expression was upregulated under hypoxia in HUVECs as determined by immunofluorescent staining. Scale bars, 50 μm. Data are presented as mean±SEM. *P<0.05 and **P<0.01 Student t test. NS indicates nonsignificant.
in HUVECs was upregulated by hypoxia as determined by immunostaining, which showed that IL-37 was localized to both cytoplasm and nucleus in HUVECs (Figure 1C). However, the expression of IL-37 in HUVECs was not affected by the principle angiogenic growth factor VEGF (Figure IC in the online-only Data Supplement). These results together established that IL-37 is expressed in human ECs and upregulated by proangiogenic hypoxic signaling.

**IL-37 Promotes EC Proliferation, Tube Formation, and Ex Vivo Sprouting of Aortae**

To address the role of IL-37 in angiogenesis, we used in vitro models of cell proliferation and tube formation in cultured human ECs. We found that supplementation of recombinant IL-37 in cell culture medium significantly stimulated HUVEC proliferation at concentrations ranging from 10 to 10 ng/mL in a dose-dependent manner, with potency comparable with that of VEGF (Figure 2A). IL-37 also stimulated HMEC-1 cell proliferation when used at 0.03 to 3 ng/mL (Figure II in the online-only Data Supplement). The biologically optimal concentration of IL-37 for EC proliferation is \( \approx 1 \) ng/mL. Similar to its effect on proliferation, IL-37 significantly promoted the formation of capillary-like structures on Matrigel, as quantified by total tubule length and branching points (Figure 2B and 2D). To verify the specificity of recombinant IL-37, the effect of IL-37 on EC proliferation were validated by blockade of IL-37 with IL-37-neutralizing antibodies from different suppliers (Figure IV in the online-only Data Supplement). We next characterized IL-37’s proangiogenic effect by assessing the effect of IL-37 on aortic sprouting ex vivo. Mouse aortic rings were prepared from wild-type mice aortae and treated with IL-37 with or without antibodies targeting IL-37 for 7 days. Sprouting from explanted aortic rings was significantly increased in the presence of IL-37, whereas the effect was significantly inhibited by IL-37 neutralizing antibodies, confirming the specificity of IL-37 (Figure 2E). These results together suggest that IL-37 is a potent proangiogenic factor.

**Effects of IL-37 on EC Migration**

Migration of ECs toward the angiogenic stimulus is an important early process in angiogenesis. To assess the effect of IL-37 on EC migration, we have determined the effect of IL-37 in a scratch-wound assay. After a scratch wound was created on the monolayer of HUVECs, increasing concentrations of IL-37 (0.1, 1, and 10 ng/mL) were added to the supernatant for 12 hours and cells were monitored 6 and 12 hours after the wound scratch. IL-37 increased wound-induced cell migration at 12 hours post wound formation when compared
with control (Figure 3A and 3B). After wounding, about 374-μm width of the wound surface was covered by migrating HUVECs treated with 1 ng/mL IL-37, whereas 225-μm width of the scratched area was covered by HUVECs treated with vehicle. In the modified Boyden chamber assay, IL-37 also induced an increase in the migrated cells to the lower chamber (Figure 3C and 3D). The optimal concentration of IL-37 to induce EC migration is ≈0.5 to 1 ng/mL, suggesting the potent effect of IL-37 on EC activation.

**IL-37 Activates Survival Signals in ECs**

To understand the signaling events induced by IL-37, we examined the major endothelial survival signals, including the extracellular signal-regulated kinase 1/2 (ERK1/2) and the serine–threonine kinase AKT, which are crucial for EC proliferation, survival, and migration. We found that IL-37 stimulated ERK1/2 and AKT activation in time-dependent manner as revealed by phosphorylation of ERK1/2 and AKT, which is consistent with our observation that IL-37 induced EC proliferation and migration. The results suggest that IL-37 promoted angiogenesis probably by activating survival signals relevant to ERK1/2 and AKT signaling (Figure V in the online-only Data Supplement). Because IL-37 was previously suggested to bind to IL-18Rα, as well as the decoy receptor IL-1R8, to gain more insight in the molecular mechanism involved in the proangiogenic effect of IL-37, we determined whether IL-37 exerts its proangiogenic function through IL-18Rα or IL-1R8 receptors with neutralizing antibodies. The result showed that antibodies against IL-18Rα or IL-1R8 receptors did not abolish the effect of IL-37 on EC proliferation and migration, suggesting that IL-18Rα and IL-1R8 are not involved in IL-37-induced proangiogenic responses (Figure VI in the online-only Data Supplement).

**IL-37 Enhances Vessel Growth In Vivo in Matrigel Plug Assay**

Having demonstrated that IL-37 promotes vascular growth in vitro and ex vivo, we then investigated the angiogenic effect of IL-37 in vivo. We have incorporated IL-37 into Matrigel and then injected the mixture subcutaneously into the middle line of the back in mice. After 7 days of implantation, IL-37 significantly stimulated Matrigel vessel growth in a dose-dependent manner as determined by hemoglobin content and vascular area, with potency comparable with that of basic fibroblast growth factor (Figure 4A–4C). To verify the vascular area was blood vessels, sections of Matrigel plugs were immunostained with CD31 and positive area was analyzed (Figure 4A and 4D). The angiogenic response to IL-37 was blocked by antibodies against IL-37 when incorporated in the
Matrigel, whereas a preimmune IgG control had no effect on IL-37-induced vessel growth (Figure VII in the online-only Data Supplement). These results are consistent with the proangiogenic effect of IL-37 on ECs in vitro and on aortic rings ex vivo.

**IL-37 Regulates Developmental Angiogenesis in Neonatal Mice**

Because our results suggest that IL-37 has a potent effect on angiogenesis in vitro and in vivo, we next investigated whether IL-37 regulates neovascularization under pathological conditions.

Figure 4. Interleukin (IL)-37 promotes angiogenesis in Matrigel plug assay in vivo. A, The angiogenic effect of IL-37 in Matrigel Plug assay. Matrigel plugs supplemented with indicated concentrations of IL-37 or mouse basic fibroblast growth factor (100 ng/mL) were subcutaneously injected into the middle line of the back in mice. Representative pictures of hematoxylin and eosin–stained sections and CD31 immunofluorescence-stained sections were shown. Scale bars, 100 μm. Accumulated analysis of hemoglobin content (mg per gram body weight; B), percentage of vascular area (C), and percentage of CD31-positive area per field (D) were shown in graphs; n=8 per group.

Data are presented as mean±SEM. **P<0.01, ***P<0.001, and ****P<0.001 Student t test. NS indicates nonsignificant.

Figure 5. Interleukin (IL)-37 promotes developmental angiogenesis. A, Neonatal mice were administrated with IL-37 (1 ng per gram body weight) from P1 to P4. Vascular area of the retina whole mounts was assessed by fluorescence microscopy. Scale bars, 500 μm. B, Vascular area from control group and IL-37-treated group was summarized; n=8. C, High-magnification images of retinal vasculature from control mice (top) or IL-37-treated mice (bottom). D, Densities of tip cells (white arrow) and branching points were quantified. Data from 8 animals were summarized in the graphs. Scale bar, 50 μm. Data are presented as mean±SEM. *P<0.05 and **P<0.01 Student t test. NS indicates nonsignificant.
conditions. The retinal vascularization is a widely used model for physiological angiogenesis because the retinal network begins to develop a network from the optic nerve after birth in neonatal mice. To determine the physiological relevance of IL-37 in promoting developmental angiogenesis, neonatal mice were treated with IL-37 (1 ng/g bodyweight) from postnatal day (P)1 to P4. Consistent with the stimulatory role of IL-37 in angiogenesis in vitro and in vivo, IL-37 significantly promoted the spreading of retinal blood vessels toward the retinal periphery at P5 as determined by the vascular area (Figure 5A and 5B). Immunofluorescence microscopy further showed that the vessel density and tip cell numbers also increased in IL-37-treated mice (Figure 5C and 5D). However, high concentration of IL-37 (15 ng/g bodyweight) inhibited the development of retinal vasculature (Figure VIII in the online-only Data Supplement).

**IL-37 Promotes Pathological Angiogenesis**

In the mouse model of OIR, hypoxia-triggered angiogenic response induces the formation of pathological neovessels, which leave the organized retinal vasculature and grow into the vitreous cavity to form unorganized, small-caliber vessels, termed neovascularization tufts. The highest extension of pathological neovascularization tufts is observed in association with plasma leakage from the neovessels at P17. Thus, neovascularization tufts are considered as the hallmark for characterization of pathological angiogenesis in the OIR model. To assess the effect of IL-37 on pathological angiogenesis, mice were intraperitoneally administrated with recombinant IL-37 at doses of 1 or 5 ng/g bodyweight, respectively, on postnatal day 12 (P12), P14, and P16. Disordered neovascular growth (tufts; B) in retina were highlighted in red and quantified. Avascular regions (C) delineated by white boundary line were quantified. Scale bars, 500 μm (lower magnification) and 100 μm (higher magnification); n=8 per group. **P<0.01 and ***P<0.001 Student t test.

Figure 6. Interleukin (IL)-37 promotes pathological angiogenesis in the mouse model of oxygen-induced retinopathy. A. Mice were administrated with PBS, 1 or 5 ng/g bodyweight of IL-37 at postnatal day 12 (P12), P14, and P16 during normoxia phase. Mice administrated with 5 ng/g bodyweight of IL-37 were treated with 5 μg/g bodyweight of control IgG or IL-37-neutralizing antibodies at P12, P14, and P16. Disordered neovascular growth (tufts; B) in retina were highlighted in red and quantified. Avascular regions (C) delineated by white boundary line were quantified. Scale bars, 500 μm (lower magnification) and 100 μm (higher magnification); n=8 per group. **P<0.01 and ***P<0.001 Student t test.
and 6B), suggesting that IL-37 significantly promotes pathological angiogenesis. The size of avascular region was similar between vehicle and IL-37-treated mice (Figure 6C).

Administration of antibodies neutralizing IL-37 abolished the proangiogenic effect of IL-37 in neovascularization tufts formation, confirming the specificity of IL-37. IL-37 did not affect retinal vasculature under normoxia conditions, suggesting that IL-37 had no effect on normal vasculature (Figure IX in the online-only Data Supplement). However, all the mice treated with IL-37 at the dose of 20 ng/g bodyweight died before P17, most probably because of hypoxia-induced vascular permeability. In addition, as IL-37 is an anti-inflammatory cytokine, to understand the effect of IL-37 on proinflammatory responses during angiogenesis, we determined the expression levels of tumor necrosis factor-α and IL-1β by quantitative polymerase chain reaction in the retinas from neonatal mice and OIR mice. The result showed that IL-1β expression in neonatal retinas was decreased in IL-37-treated mice, and the expression levels of both tumor necrosis factor-α and IL-1β were decreased in OIR retinas from IL-37-treated mice, suggesting that IL-37 inhibited inflammatory cytokine production in retinal angiogenesis (Figure X in the online-only Data Supplement). These results suggest that IL-37 is a potent proangiogenic cytokine that promotes pathological angiogenesis.

**Discussion**

Our results identified IL-37 as a novel and potent angiogenic cytokine in both pathological and physiological settings. Although IL-37 is emerging as an important anti-inflammatory cytokine, which mainly function intracellularly via interaction with nuclear transcription factor such as Smad3,10 IL-37 is secreted as an extracellular cytokine under some conditions. For example, the secretion of mature IL-37 from RAW cells with transgenic IL-37 overexpression and PBMCs is induced by ATP, depending on caspase activity. Secretion of IL-37 from dendritic cells can be induced by antibodies against CD94, HLA-I, and intercellular adhesion molecule 1. These lines of evidence suggest the possibility that IL-37 functions as an extracellular cytokine. Indeed, as a secreted cytokine, IL-37 is upregulated in the serum of patients with systemic lupus erythematosus, Guillain–Barré syndrome, hepatitis B virus infection, and amyotrophic lateral sclerosis.14,15,16,17 Here, we observed that IL-37 is expressed by ECs, suggesting angiogenic function of IL-37. Importantly, IL-37 expression and secretion is induced by hypoxia, a well-known proangiogenic conditions that stimulates angiogenesis and vascular alterations.25 Knockdown of HIF-1α expression inhibited hypoxia-induced upregulation of IL37 mRNA and IL-37 protein levels, indicating that IL-37 expression and secretion are regulated by proangiogenic hypoxic signaling. Interestingly, the principle angiogenic factor VEGF did not have any effect on the expression of IL-37.

To our knowledge, the role of IL-37 on angiogenesis has not been investigated before. On the basis of our results, amounts as low as 10 pg/mL of IL-37 induced EC activation, and low concentration of IL-37 exhibit potent activity in vivo. Aortic rings excised from mice exhibited enhanced vessel sprouting after treatment with IL-37 ex vivo. The increase in vessel sprouting may be because of the effect of IL-37 on EC proliferation and migration as shown by our in vitro studies. The increase in vessel sprouting was specifically inhibited by antibodies against IL-37, suggesting the specificity of the angiogenic activity of IL-37. We have further demonstrated the proangiogenic effect of IL-37 in mouse models in vivo. IL-37 increased vessel growth in the Matrigel plug in a dose-dependent manner. When incorporated at the concentration of 100 ng/mL, the proangiogenic effect of IL-37 was as potent as basic fibroblast growth factor. We further demonstrated that IL-37 significantly promoted neovascularization in developmental and pathophysiological angiogenesis using the mouse model of retinal development and OIR. The mechanism by which IL-37 promotes angiogenesis is under future investigation. Mice carrying transgenic or transient expression of human IL-37 exhibited reduced inflammation in models of hepatitis, colitis, and psoriasis.26–28 However, IL-37 transgenic mice have not been reported to exhibit severe vascular abnormalities and embryonic lethality, suggesting that intracellular IL-37 is probably retained intracellularly and was not able to exert its proangiogenic effect in IL-37 transgenic mice.

Survival signals conducted by ERK1/2 and AKT have been recognized to be critical for endothelial activation and viability.19,20 We found that IL-37 stimulated ERK1/2 and AKT activation, suggesting that IL-37 promoted angiogenesis probably by activating survival signals relevant to ERK1/2 and AKT signaling. Previous studies have revealed that IL-37 binds to IL-18 receptor as well as IL-1R8.18,21–23 Unexpectedly, IL-18Rα or IL-18 antibodies did not block the effect of IL-37 on EC proliferation and migration, suggesting that IL-18Rα and IL-1R8 are not involved in IL-37-induced proangiogenic responses. Therefore, the identification of the putative receptor that mediates the proangiogenic effect of IL-37 remains unclear and is currently under investigation in our laboratory.

The result that IL-37 suppressed proinflammatory cytokine production in retina angiogenesis raises the question whether IL-37 modulates angiogenic response by modulating proinflammatory reaction. However, many proinflammatory cytokines released during inflammation are potent activators of ECs.29,30 For example, it has been suggested that tumor necrosis factor-α directly regulates angiogenesis through increasing ECs survival and vessel maintenance.31 VEGF is produced in response to proinflammatory cytokines including tumor necrosis factor-α and IL-1.32 These studies thus argue against the possibility that IL-37 promotes angiogenesis through modulating inflammatory responses. Furthermore, our study showed that IL-37 directly promotes EC proliferation, migration, and tubule formation in vitro, suggesting that IL-37 directly induces angiogenic response through activating ECs.

The dose-dependent effect of IL-37 might be because of several reasons. First, in angiogenic assays, the proangiogenic effects of many angiogenic factors were biphasic. For example, PAI-1 is proangiogenic at physiological concentrations but becomes antiangiogenic at higher concentrations.33 IL-8 induces chemotaxis and proliferation of ECs at low concentrations, but its activity was reduced at
higher concentrations.34 Another study showed that statins promoted angiogenesis at low concentrations, but inhibited angiogenesis at high concentrations both in vitro and in vivo.35 Interestingly, angiostatic factors, such as IP-10 and thrombospondin, were reported to suppress angiogenesis at low concentrations but induce EC chemotaxis at high concentrations.6–38 The underlying basis of the biphasic functions of angiogenic factors remains to be elucidated. Second, the physiological concentration of IL-37 is typically less than 100 pg/mL in serum.35 It is thus expected that high doses of IL-37 over the physiological range may cause EC cytotoxicity in experiments.

The identification of IL-37 as a proangiogenic cytokine has important clinical implications. Notably, administration of recombinant IL-37 ameliorates myocardial ischemic injury or hepatic ischemia/reperfusion injury during acute phase in models.39,40 According to our results, it is speculated or hypothesized that IL-37 ameliorates myocardial ischemic injury through promoting angiogenesis at low concentrations but induce EC chemotaxis at high concentrations.6–38 Further investigations are in progress to uncover the regulatory role of IL-37 in vascular functions under physiological and pathological settings.

Notably, IL-37 is detected in tumor cells of breast carcinoma and infiltrating plasma cells in colon carcinoma,18 suggesting possible involvement of IL-37 in the pathogenesis of cancer. Thus, further goals are to determine whether blockade of IL-37 as an intriguing strategy for manipulating neovascularization in angiogenesis-related diseases.

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Disclosures
None.

References


Interleukin 37 (IL-37) is a newly identified cytokine of the IL-1 family, some members of which are important in inflammation and angiogenesis. However, the function of IL-37 in angiogenesis has remained unknown. We report that IL-37 was expressed in endothelial cells and upregulated under hypoxic conditions. IL-37 enhanced endothelial cell proliferation, capillary formation, migration, and promoted aortic ring sprouting with potency comparable with that of vascular endothelial growth factor. In mice, IL-37 enhanced angiogenesis in Matrigel plug assay and promoted neovascularization in oxygen-induced retinopathy and in neonatal retinal vasculature. The identification of IL-37 as a proangiogenic cytokine in pathophysiological settings has important clinical implications. Our results implicate that IL-37 probably contribute to angiogenesis-related diseases, such as diabetic retinopathy, cancer, and cardiovascular diseases. Thus, targeting IL-37 would present an intriguing therapeutic strategy for manipulating neovascularization and disease progression in angiogenesis-related diseases.
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Supplemental Figures

Supplemental Figure I. IL-37 expression in endothelial cells. (A) IL-37 expression in HUVECs and HMEC-1 cells as determined by ELISA. n = 5. (B) Hypoxic conditions (0.1% O₂) stimulated Il37 mRNA expression in HUVECs as determined by real-time PCR. n = 5. (C) VEGF did not affect IL-37 expression in HUVECs. HUVECs were pre-starved overnight and treated with 20 ng/mL of VEGF and IL-37 in cell lysates was determined by ELISA. Data are presented as mean ± SEM (n = 4 per group).
**Supplemental Figure II.** Knockdown of *HIF1A* inhibited hypoxia-induced upregulation of IL-37 expression in HUVECs. (A) HUVECs were transfected with 100 nmol/L control (Ctrl) siRNA or *HIF1A* siRNA and *HIF1A* mRNA level was quantified by quantitative PCR. *n* = 5 per group. Control or *HIF1A* siRNA transfected HUVECs were cultured under normoxic or hypoxic conditions. *IL37* mRNA level was quantified by quantitative PCR (*n* = 5) (B) and IL-37 protein level was determined by Western Blot (C). Data were presented as mean ± SEM. **P* < 0.01 Student's *t* test.
Supplemental Figure III. Effect of IL-37 on HMEC-1 cell proliferation. After incubated with indicated concentrations of IL-37, cell proliferation was measured using BrdU ELISA assay. VEGF was used at 10 ng/mL. n = 5. *P < 0.05. **P < 0.01, ***P < 0.001.
Supplemental Figure IV. Specific pro-angiogenic activity of IL-37. The effect of IL-37 from different suppliers on HUVEC proliferation was examined. IL-37 from Life Technology (LT), R&D Systems (R&D) was neutralized with 20 µg/mL anti-IL-37 antibodies (α-IL-37) from either R&D Systems of Abcam. *n* = 5 each group. Data are presented as mean ± SEM. **P<0.01.
Online Figure V. Effects of IL-37 on EC survival signaling. (A) HUVECs were treated with 1 ng/mL of IL-37 and phosphorylation of ERK and AKT was examined by Western Blot. (B) Densitometric analysis of p-ERK/ERK and p-AKT/AKT was shown (n = 3). Data are presented as mean ± SEM. *P< 0.05, **P< 0.01.
Online Figure VI. IL-18 Rα or IL-1R8 did not mediate the effect of IL-37 on endothelial cell proliferation and migration. (A) HUVECs were treated with 1 ng/mL of IL-37 in the absence or presence of IL-18Rα (α- IL-18Rα, 10 µg/mL) or IL-1R8 (α- IL-1R8, 10 µg/mL) neutralizing antibodies and cell proliferation was measured using BrdU ELISA assay (n = 5 per group). (B) HUVECs were allowed to migrate from the upper chamber to the lower chamber in modified Boyden Chamber Assay. IL-37 (0.5 ng/mL), α- IL-18Rα and α-IL-1R8 (10 µg/mL) were added to the lower chamber as indicated. Migrated cells per filed were quantified. Data were presented as mean ± SEM (n = 4 per group). NS, not significant.
Supplemental Figure VII. IL-37 neutralizing antibodies blocked the effect of IL-37 in Matrigel plug assay. (A) Representative images of hematoxylin and eosin-stained sections of Matrigel plugs incorporated with 50 ng/mL IL-37 together with 10 µg/mL IgG or 10 µg/mL α-IL-37 neutralizing antibodies. 7 days after implantation, the representative pictures of total Matrigel plug were shown on top right insets. Immunofluorescent images of sections stained with anti-CD31 antibodies were shown in lower panel. Scale bars, 100 µm. (B) Accumulated analysis of hemoglobin content, percentage of vascular area and percentage of CD31-positive area per field were shown in graphs. n = 8 per group. Data are presented as mean ± SEM. *P< 0.05, **P< 0.01 and ***P< 0.001.
Supplemental Figure VIII. Effects of high concentration of IL-37 on retinal vasculature. Neonatal mice were intraperitoneally injected with IL-37 at the concentration of 15 ng/gram bodyweight from P1 to P4. Littermate controls were administrated with the same volume of PBS. The whole retina mounts were isolated at P5 and stained with FITC-conjugated Isolectin B4. (A) Representative retinal vasculature. Scale bar, 500 µm. (B) Vascular area of the retina whole mounts from PBS or IL-37 treated neonatal mice. n = 8. Data are presented as mean ± SEM. *P< 0.05.
Supplemental Figure IX. Effect of IL-37 on retinal vasculature under normoxic conditions. Mice were kept at room air and treated with either PBS or 5 ng per gram bodyweight of IL-37 on P12, P14 and P16. Mouse retinas were isolated at P17 and stained with FITC-conjugated Isolectin B4. Scale bar, 500 µm.
**Supplemental Figure X.** Effect of IL-37 on pro-inflammatory cytokine production in retinal angiogenesis. (A) mRNA levels of *Tnfa* and *Il1b* in retinas of postnatal day 5 (P5) neonatal mice treated with PBS or 1 ng/g bodyweight IL-37 from P1 to P4. *n*=4 (B) mRNA levels of *Tnfa* and *Il1b* in P17 retinas of OIR mice treated with PBS or 1 ng/g IL-37 at postnatal day 12 (P12), P14 and P16 during normoxia phase. *n*= 4. Expression levels were normalized to retinas of untreated P17 mice. Data are presented as mean ± SEM. *P*< 0.05, **P**< 0.01.
Materials and Methods

Reagents
Recombinant human IL-37 was purchased from Life Technologies (Thermo Fisher Scientific, Gaithersburg, MD) or R&D Systems (Minneapolis, MN). Antibodies against IL-37, IL-18Rα, IL-1R8 were purchased from R&D Systems (Minneapolis, MN) and Abcam (Cambridge, MA). Antibodies against ERK1/2, phosphorylated ERK1/2, AKT, phosphorylated AKT and β-actin were purchased from Cell Signaling Technology (Beverly, MA). Human recombinant human VEGF-A were obtained from R&D systems (Minneapolis, MN).

Animals
C57BL/6 mice for oxygen-induced retinopathy model were purchased from Experimental Animal Centre of Sun Yat-sen University of Medical Science (Guangzhou, China). Care, use and treatment of all animals were in strict agreement with the guidelines of the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Visual Research and approved by the institutional animal care and use committee in Zhongshan Ophthalmic Center. For aortic ring experiments and retinal vasculature, C57BL/6J mice were from Shanghai Laboratory Animal Center (SLAC, Shanghai, China). All experimental protocols were approved by the Animal Care and Use Committee of Tongji University.

Cells and culture
HUVECs were purchased from ScienceCell and maintained in ECM medium (ScienCell, Carlsbad, CA) according to company's instructions. Specifically, ECM medium was supplemented with 5% FBS and endothelial cell growth supplement ((ECGS, Cat. No. 1052). Cells used for experiments were between passages 3 to 6. HMEC-1 cells were obtained from Centers for Disease Control and Prevention (Atlanta, GA) and cultured in MCDB-131 (Invitrogen, Carlsbad, CA) supplemented with 10 ng/mL epidermal growth factor (Peprotech, Rocky Hill, NJ), 10 ng/mL hydrocortisone (Sigma, St. Louis, MO), and 10% fetal bovine serum (GIBCO, Grand Island, NY). All cells were cultured at 37 °C in a humidified atmosphere of 5% CO2-95% air.

HIF-1α siRNA knockdown
For HIF-1α knockdown, siRNAs oligonucleotides targeting HIF1α (#1, target sequence 5′-auggaaauauauacugcgniua-3′; #2, target sequence 5′-aggaagaacuaangaacuauaa-3′) and negative control siRNA were purchased from Qiagen. HUVECs were transfected with 100 nM control siRNA or 100 nM siHIF1A comprised 50 nM of the two antisense sequences using Lipofectamin RNAiMAX (Invitrogen, Carlsbad, CA) according to manufacturer's instructions.

mRNA isolation and qPCR
Cells or mouse retinas were lysed with TRIzol (Sigma, St. Louis, MO), and mRNA was extracted and reverse transcribed according to manufacturer's directions (Qiagen, Valencia, CA). Probes for human II37 were 5′-CTCCTGCGGGGTCCTTAAAG-3′ (Forward) and
5'-TACAATTGCAGGAGGTGCAG-3' (Reverse). Probes for human β-actin were 5'-CCAGGGCCTATGGTAGGCA-3' (Forward) and 5'-CCAGGGCCTATGGTAGGCA-3' (Reverse). Probes for mouse TNFa were 5'-TGAGCACAGAAAGCATGATCCG-3' (Forward) and 5'-GTAGACAGAAAGCGTGGTGCG-3' (Reverse). Probes for mouse IL1b were 5'-TAATGAAAGACGGCACACCCAC-3' (Forward) and 5'-CGAAGGTGGAAGAGTGGGAGTTG-3' (Reverse). Probes for mouse Gadph were 5'-GAAGGTGGAAGAGTGGGAGTTG-3' (Forward) and 5'-CGAAGGTGGAAGAGTGGGAGTTG-3' (Reverse).

**Western Blot**
HUVECs lysates were prepared using RIPA buffer and phosphatase and protease inhibitor cocktail (Roche, Mannheim, Germany). Proteins were separated using SDS-PAGE and transferred to nitrocellulose membranes. Proteins were detected by indicated antibodies. For hypoxia experiment, cells were seeded in 24-well plates and cultivated in normal conditions for 24 h and then subjected to 0.1% O2 condition.

**Enzyme-linked Immunosorbent Assay (ELISA)**
IL-37 in supernatants was determined with ELISA kit (R&D Systems, Minneapolis, MN) according to supplier's instructions. For hypoxia experiment, cells were seeded in 24-well plates and cultivated in normal conditions for 24 h and then subjected to 0.1% O2 condition.

**Immunostaining**
The expression of IL-37 was assessed by immunohistochemistry on vasculitis and colon carcinoma tissue sections. Briefly, tissue sections were deparaffinized, quenched with H2O2. Antigens were retrieved using retrieval solution (Dako) according to company's instructions. Sections were incubated with primary antibodies at 4 °C overnight, followed by incubation with secondary antibodies. Sections were counterstained with hematoxylin. For immunofluorescence studies, frozen sections of Matrigel plugs were incubated overnight with anti-CD31 antibodies (Biolegend, San Diego, CA), followed by incubation with fluorescein-conjugated secondary antibodies. Sections were mounted and examined with fluorescent microscopy. For Immunofluorescent staining of cultured HUVECs, cells were fixed, blocked with PBS supplemented with 5% BSA and incubated with 2 µg/mL α-IL-37 antibodies. Cells were then labeled with a fluorescein TRITC-conjugated secondary antibodies for 1h at room temperature, rinsed in PBS and examined with fluorescent microscopy.

**Proliferation assay**
For the effect of IL-37 on HUVECs proliferation, 1×10^4 HUVECs in 100 µl of ECM were seeded in 96-well plates and maintained ECM containing 5% FBS without supplemented growth factors unless otherwise indicated. Cells were stimulated with IL37 or VEGF at different concentrations (0, 0.001, 0.01, 0.1, 1, 10 and 100 ng/mL) for 24 hours. Anti-IL-18Rα or anti-IL-1R8 neutralizing antibodies were used at 10 µg/mL. Cell proliferation was determined by BrdU ELISA kit (Abcam, Cambridge, MA) according to company's instructions. The statistical significance was determined by Student's t-test.
Endothelial wound healing assay
Before the experiment, HUVECs were starved in serum-free medium overnight without supplementary growth factors. A scratch-wound was created on the monolayer of HUVECs with a 200 µl pipette tip. IL-37 was added to the medium at indicated concentrations. 2.5 µg/mL cytosine arabinoside (Sigma, St. Louis, MO) was added to the cell to block cell proliferation. Endothelial cell migration was recorded with microscope 6 and 12 hours after wound scratch. The width of the wound was measured by Photoshop.

Modified Boyden Chamber migration assay
HUVECs were serum starved in serum-free medium without supplementary growth factors overnight in ECM. Transwell inserts (8 µl pore size, Corning, Corning, NY) were coated with 10 µg/mL of fibronectin (Sigma, St. Louis, MO) and 5×10⁴ HUVECs in 100 µl of ECM were added to the upper chamber. The lower chamber were filled with 600 µl of ECM with indicated concentration of IL-37. Anti-IL-18Rα or anti-IL-1R8 neutralizing antibodies were used at 10 µg/mL. Cells were allowed to migrate at 37°C in CO₂ incubator for 10 hours. Non-migrating cells were removed with a cotton swab and migrated cells were fixed, stained with 1% crystal violet and counted in three different fields under microscope.

Matrigel tube formation assay
HUVECs were cultured in ECM containing 5% FBS without supplementary growth factors overnight. 1×10⁴ HUVECs in 100 µl of ECM were seeded on growth factor-reduced Matrigel (BD Biosciences, Bedford, MA) in 96-well plate. Media was supplemented with indicated concentrations (0, 0.1, 1, 10 and 100 ng/mL) of IL-37 and VEGF for 12 hours of different concentrations (0, 0.1, 1, 10 and 100 ng/mL). After 6 hours, the capillary-like structures were recorded with microscopy and tube length and branching points were measured with Photoshop.

Matrigel plug assay
Sterile growth factor reduced Matrigel (BD Biosciences, Bedford, MA) supplemented with IL-37 (5 ng/mL, 25 ng/mL, 50 ng/mL, 100 ng/mL) and mouse bFGF (100 ng/mL) was subcutaneously into the middle line of the back in mice. Antibodies against IL-37 or IgG control (10 µg/mL) were mixed together with IL-37 in the Matrigel as indicated. After 7 days, mice were anesthetized and Matrigel plugs were carefully pulled out after implantation and divided into 2 blocks. One Matrigel plug was weighed and digested with tissue homonizer and hemoglobin content was determined by Drabkin method (Sigma-Aldrich, St. Louis, MO). The other plug was fixed in 4% formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin and then quantified under microscope. Vascular area was determined as the total lumen area of vessels, which are filled with erythrocytes. The percentage of vessel lumen area in 5 random fields per section was quantified. Immunofluorescent staining of sections was performed with anti-CD31 antibodies was performed to identify endothelial cells.

Aortic ring angiogenesis assay
Rings were sliced from aortae of 4-week old wild-type mice and sectioned into ~0.5mm pieces. Aortic rings were embedded in a 96-well plate containing collagen I gel (Millipore, Bedford, MA). The embedded rings were fed with M199 culture medium supplemented with 2.5% FBS. VEGF (5 ng/mL), IL-37 (1 ng/mL), anti-IL-37 antibodies (10 µg/mL) and IgG (10 µg/mL) were added to the culture medium as indicated. Growth medium was changed every two days. The aortic ring sprouting was photographed and the degree of sprouting was graded from 0 (least positive) to 5 (most positive) in a double-blinded manner.

Mouse model of OIR
Neonatal mice and their nursing mothers were placed in a 75% oxygen supply chamber from P7 to P12. Mice were returned to normal oxygen supply (21%) at P12. Mice were injected with PBS or IL-37 at 1, 5, 20 ng/gram bodyweight at P12, P14 and P16. Mice administrated with 5 ng/g bodyweight of IL-37 were treated with 5 µg/g bodyweight of control IgG or IL-37-neutralizing antibodies at P12, P14 and P16. At P17, the whole retinal mounts were isolated and stained with Isolectin B4 (Sigma). Neovascular region (tufts) and avascular area were analyzed using Image J. Significant differences were determined using Student's t test.

Mouse retinal vascularization
Neonatal mice were intraperitoneally injected with IL-37 at the concentration of 1 ng/gram bodyweight or 15 ng/gram bodyweight from P1 to P4. Littermate controls were administrated with the same volume of PBS. The whole retina mounts were isolated at P5 and stained with FITC-conjugated Isolectin B4. Retinal vasculature was assessed by fluorescent microscopy and vascular area was analyzed with Image J.

Data and statistical analyses
Data are presented as means ± SEM. Prism software was used for statistical analyses. Significant differences between paired samples were analyzed with the two-tailed Student's t test. A P value of less than 0.05 was considered significant for all analyses.