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IL-17 Contributes to Angiogenesis in Rheumatoid Arthritis

Sarah R. Pickens,* Michael V. Volin,† Arthur M. Mandelin, II,* Jay K. Kolls,‡ Richard M. Pope,*,§ and Shiva Shahrara*

Angiogenesis is an early and a critical event in the pathogenesis of rheumatoid arthritis (RA). Neovascularization is dependent on endothelial cell activation, migration and proliferation, and inhibition of angiogenesis may provide a novel therapeutic approach in RA. In this study, we document a novel role of IL-17 in mediating angiogenesis. Local expression of IL-17 in mouse ankles increases vascularity. We further demonstrate that IL-17 is angiogenic by showing its ability to promote blood vessel growth in Matrigel plugs in vivo. Additionally, IL-17, in concentrations present in the RA joint, induces human lung microvascular endothelial cell (HMVEC) migration mediated through the PI3K/AKT1 pathway. Furthermore, suppression of the PI3K pathway markedly reduces IL-17-induced tube formation. We also show that both IL-17-induced HMVEC chemotaxis and tube formation are mediated primarily through IL-17 receptor C. Neutralization of either IL-17 in RA synovial fluids or IL-17 receptor C on HMVECs significantly reduces the induction of HMVEC migration by RA synovial fluid. Finally, RA synovial fluid immunneutralized with anti–IL-17 and antivasculature endothelial growth factor does not reduce HMVEC migration beyond the effect detected by immunodepleting each factor alone. These observations identify a novel function for IL-17 as an angiogenic mediator in RA, supporting IL-17 as a therapeutic target in RA. The Journal of Immunology, 2010, 184: 3233–3241.

These mice are crossed with IL-17–deficient mice (13), suggesting that Th-17 cell differentiation may be the reason for the arthritis. IL-17 is found in rheumatoid arthritis (RA) synovial tissue and fluid, and the percentage of Th-17 cells is significantly higher in RA synovial fluid compared with RA or normal peripheral blood (3, 14). IL-17 may play an important role in the pathogenesis of RA by inducing the production of proinflammatory cytokines and chemokines from RA synovial tissue fibroblasts including IL-6, CXCL8/IL-8, CCL2/MIP-3α, CXCL1/growth-related oncogene-α, and CXCL2/growth-related oncogene-β (15–17). IL-17 is also capable of activating macrophages to expression of IL-1, TNF-α, cyclooxygenase 2, PGE2, and matrix metalloproteinase-9 (18–20). We recently demonstrated that IL-17 is directly chemoattractive for monocytes (21), and others have shown that IL-17 activates neutrophil recruitment through the stimulation of chemokines such as CXCL1 in rats and CXCL8/IL-8 in humans (22, 23).

Angiogenesis, the development of new capillaries, is involved in leukocyte ingress into the synovium during the development and progression of RA (24, 25). However, the role of IL-17 in angiogenesis is undefined. There are contradictory results regarding the role of IL-17 on tumor development and angiogenesis. In mice, tumors transfected with IL-17 demonstrated markedly less growth compared with control tumors (26). Additionally, tumor growth and lung metastasis were increased in IL-17–deficient mice (27), suggesting that IL-17 inhibits tumor development and neovascularization. Others have shown that IL-17 increases blood vessel development in rat cornea and tumor vascularity in animal models, indicating that IL-17 may be important for angiogenesis (28). However, there are also data to suggest that IL-17 alone is unable to induce angiogenesis but can indirectly mediate human lung microvascular endothelial cell (HMVEC) growth by promoting the mitogenic activity of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and hepatocyte growth factor (29, 30).

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Abbreviations used in this paper: Ad, adenovirus; bFGF, basic fibroblast growth factor; EBMM, endothelial basal medium; Endo, endothelial cell; FGF, fibroblast growth factor; HEK, human embryonic kidney; HMVEC, human lung microvascular endothelial cell; IL-17RA, IL-17 receptor A; IL-17RC, IL-17 receptor C; p, phospho; RA, rheumatoid arthritis; VEGF, vascular endothelial growth factor.
binding to its receptors or indirectly by inducing proangiogenic factors from cells present in the RA synovium.

In the current study, we have therefore evaluated the role of IL-17 in HMVEC migration and tube formation as well as blood vessel development. Our results demonstrate that local expression of IL-17 increases vascularity in mouse ankle joints. Using a Matrigel plug assay, we demonstrate that IL-17 enhances blood vessel formation in vivo. Although HMVECs express both IL-17RA and RC, IL-17-induced HMVEC chemotaxis and tube formation are mediated by binding primarily to IL-17RC and activation of the PI3K pathway. We report that RA synovial fluid-mediated endothelial migration is significantly reduced by IL-17 and/or IL-17RC neutralization. Finally, we show that IL-17 and VEGF neutralization in RA synovial fluid does not significantly reduce HMVEC migration beyond the effect of one factor alone. Hence, therapy directed against IL-17 may reduce leukocyte migration by inhibiting angiogenesis in RA.

Materials and Methods

Study protocol for local expression of IL-17 in mouse ankle joints

The animal studies were approved by the Northwestern University Institutional Review Board. Adenovirus (Ad) constructed as reported previously to contain an IL-17 payload was provided by J. K. Kolls (Louisiana State University Health Science Center, New Orleans, LA) (31). Four- to six-week-old C57BL/6 mice were injected intra-articularly with 10⁵ PFU Ad-IL-17 or Ad-CMV control. Ankle circumference and articular index score were determined on days 4 and 10 post-Ad-IL-17 injection and compared with the control group (data not shown). Ankles were harvested on day 10 post-Ad-IL-17 injection for histological studies. Levels of IL-17 were quantified by ELISA on days 4 and 10 from ankles treated with Ad-IL-17 or Ad-CMV control.

Abs and immunohistochemistry

Mouse ankles were decalcified, formalin fixed and paraffin embedded, and sectioned in the pathology core facility of Northwestern University. Mouse ankles were immunoperoxidase stained using Vector Elite ABC Kits (Vector Laboratories, Burlingame, CA), with diaminobenzidine (Vector Laboratories) as a chromogen. Slides were deparaffinized in xylene for 20 min at room temperature, followed by rehydration by transfer through graded alcohols. Ags were unmasked by first incubating slides in boiling citrate buffer for 15 min, followed by type II trypsin digestion for 30 min at 37°C. Endogenous peroxidase activity was blocked by incubation with 3% H₂O₂ for 5 min. Nonspecific binding of avidin and biotin was blocked using an avidin/biotin blocking kit (Vector Laboratories). Nonspecific binding of Abs to the tissues was blocked by pretreatment of tissues with diluted normal goat serum. Tissues were incubated with rabbit polyclonal Ab to von Willebrand factor (1/100 dilution; Serotec, Oxford, U.K.) or a rabbit IgG control Ab (1/20 dilution; Coulter, Fullerton, CA). Slides were counterstained with Harris hematoxylin and treated with lithium carbonate for bluing. Each slide was evaluated by a blinded observer (32-35) (M.V.V.). Tissue sections were assigned a vascular score representing the number of blood vessels in each section. A semiquantitative score of 1 represented a tissue with few blood vessels, whereas a score of 4 represented a highly vascularized tissue. Scored data were pooled, and the mean ± SEM was calculated in each data group (n = 5).

Matrigel plug assay in vivo

To examine the effect of IL-17 on angiogenesis in vivo, we used a Matrigel plug assay. Four- to six-week-old C57BL/6 mice were injected i.s.c. in the dorsal area with 500 μl Matrigel. Matrigel plus PBS served as negative control, Matrigel containing bFGF (20 ng/ml) served as positive control, and experimental conditions included Matrigel with mouse recombinant IL-17 (2 μg). After 10 d, mice were sacrificed, Matrigel plugs were carefully dissected out, and surrounding connective tissue was removed, and plugs were analyzed for vascularity by hemoglobin measurement or by histology. For hemoglobin measurement, plugs were weighed by placing them into 1 ml preweighed double-distilled H₂O and then homogenized for 5–10 min on ice and spun. Using methemoglobin, serial dilutions were prepared to generate a standard curve from 70 to 1 μg/dl (36, 37). Fifty microliters of supernatant or standard was added to a 96-well plate in duplicate, and 50 μl tetranitromethane was added to each sample. The plate was allowed to develop at room temperature for 15–20 min with gentle shaking, and the reaction was terminated with 150 μl 2 N H₂SO₄ for 3–5 min. Absorbance was read with an ELISA plate reader at 450 nm. To calculate hemoglobin concentrations, the values (grams per deciliter) were normalized to the weights of the plugs (gram) (36, 37). On day 10, IL-17 concentrations were quantified in Matrigel plugs harvested from the IL-17–treated group using ELISA.

For histology, plugs were embedded in paraffin and sectioned in the pathology core facility of Northwestern University. Histology slides from different groups were examined by H&E and Masson’s trichrome staining (37, 38) and scored by a blinded observer (A.M.M.) based on a scale of 0–4, where 0 = no structures; 1 = tubules without lumina; 2 = tubules with lumina only; 2 tubules with multilayer wall, and 4 = tubules with multilayer walls with connective tissues surrounding them.

HMVEC chemotaxis

To examine chemotaxis, HMVECs were incubated in endothelial basal medium (EBM) with 0% FBS and no growth factors for 2 h before use. HMVECs (1.25 × 10⁵ cells/25 μl EBM with 0.1% FBS) were then placed in the bottom wells of a 48-well Boyden chemotaxis chamber (NeuroProbe, Cabin John, MD) with gelatin-coated polycarbonate membranes (8-μm pore size; Nucleopore, Pleasant, CA) (37, 39). The chambers were inverted and incubated at 37°C for 2 h, allowing endothelial cell attachment to the membrane. The chamber was reinserted, and PBS, positive control VEGF (10 ng/ml; R&D Systems, Minneapolis, MN), or IL-17 at varying concentrations from 0.001 to 100 ng/ml (R&D Systems) was added to the upper wells, and the chamber was further incubated for 2 h at 37°C. The membranes were then removed, fixed, and stained with the Protocol HEMA 3 stain set (122-911; Fisher Scientific, Waltham, MA). Readings represent the number of cells migrating through the membrane (the average of three high-power ×40 fields/well, averaged for each triplicate of wells). To test specificity of IL-17–induced HMVEC migration, HMVEC chemotaxis was examined with heat-inactivated IL-17 (10 and 50 ng/ml incubated at 100°C for 15 min) or IL-17 neutralized by anti–IL-17 Abs or IgG control (10 μg/ml for 1 h in 37°C) (R&D Systems) (21). To examine for chemokinesis, a series of checkerboard experiments were performed by placing increasing concentrations of IL-17 (0, 0.01, 1, and 10 ng/ml) together with HMVECs in the bottom wells with different concentrations of IL-17 in the top wells. To determine which IL-17 receptors are important for IL-17 HMVEC migration, Abs to IL-17RA (R&D MAB177; 10 μg/ml), IL-17RC (R&D AF2269; 10 μg/ml), both Abs, or IgG control were added to HMVECs, incubated for 1 h at 37°C, and further incubated for 2 h in the Boyden chamber with IL-17 (50 ng/ml). To define which signaling pathway(s) mediated IL-17–induced HMVEC chemotaxis, HMVECs were incubated with inhibitors to PI3K (LY294002; 1 and 5 μM), ERK (PD98059; 1 and 5 μM), and JNK (SP600125; 1 and 5 μM), or DMSO for 2 h in the Boyden chamber with IL-17 (50 ng/ml). HMVEC chemotaxis induced by RA synovial fluids was examined following 1-h incubation (37°C) of fluids with control IgG or neutralizing anti–IL-17 Abs (10 μg/ml). The fluids were diluted 1/20 in HBSS, 1/20 lower containing RBCs to the top wells (21). To examine whether IL-17 receptors are involved in RA synovial fluid-induced HMVEC chemotaxis, HMVECs were incubated with Abs to IL-17RA and RC (10 μg/ml) or both Abs, as well as IgG control (1 h at 37°C), before adding the RA synovial fluid to the top wells (21). HMVEC chemotaxis was performed to examine the synergistic effect of IL-17 and/or VEGF in RA synovial fluid induced HMVEC migration. Chemotaxis induced by RA synovial fluids was examined following incubation of fluids (diluted 1/20) (n = 8 fluids) with control IgG, anti–IL-17, anti-VEGF, or both Abs (10 μg/ml) for 1 h prior to performing the assay.

Quantification of IL-17RA and RC expression on HMVECs

Skin and lung HMVECs, HUVECs, and human embryonic kidney (HEK) 293 were cultured in EGM-2, EGM, and 10% DMEM, respectively. Total cellular RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA) from all different cell types. Subsequently, reverse transcription and real-time RT-PCR were performed to determine IL-17RA and IL-17RC expression level as described previously (14, 21, 40). Relative gene expression was determined by the ΔΔCt method, and results were expressed as fold increase above levels detected in HEK 293 cells.

Characterization of IL-17 signaling pathways in HMVECs

HMVECs (passages 3–8) were grown to 80% confluence in EGM-2 MV bullet kit (Lonza, Walkersville, MD). Cells were then switched to EGM-MV (EBM plus SingleQuots of growth supplements) for 2 d and were incubated in EBM with 0% FBS for 2 h prior to treatment. Cells were then untreated or treated with IL-17 (50 ng/ml) for 15–75 min. Cell lysates were examined by Western blot analysis, as described previously (21, 40, 41). Blots were probed with phospho (p)-AKT, pERK, and pJNK (1/1000 dilution; Cell Signaling Technology, Beverly, MA) overnight and after stripping were probed with AKT, ERK, and JNK (1/3000 dilution; Cell Signaling Technology) overnight.
**HMVEC tube formation assay**

To perform the Matrigel tube formation assay, BD Matrigel Matrix (BD Biosciences, Bedford, MA; 50 μl) was polymerized for 30 min at 37°C in a 96-well plate. To determine which IL-17 receptors play a role in IL-17–induced HMVEC tube formation, trypsinized HMVECs were resuspended (4 × 10^5 cells/ml) in EBM without growth factors with 2% FBS and incubated with Abs to IL-17RA, IL-17RC, both IL-17RA and RC or IgG for 45 min at 37°C. Cells were then added to polymerized Matrigel, IL-17 (50 ng/ml) was added to the wells, and the plate was incubated for 16 h at 37°C. To examine which signaling pathways contribute to IL-17–mediated HMVEC tube formation, cells were incubated with inhibitors to PISK (LY294002; 1 and 5 μM), ERK (PD98059; 1 and 5 μM), JNK (SP600125; 1 and 5 μM), or DMSO for 45 min at 37°C prior to adding to polymerized Matrigel. As above, IL-17 (50 ng/ml) was then added to the wells, and the plate was incubated for 16 h at 37°C. Each condition was performed in triplicate, fibroblast growth factor (FGF) (20 ng/ml) was used as a positive control, and PBS was used as a negative control. Following incubation, culture medium was removed, and cells were washed with HBSS. Thereafter, tube formation was quantified using calcein-AM fluorescent dye (BD Biosciences) diluted with HBSS and DMSO, according to the manufacturer’s instructions, for 30 min. Subsequently, the plates were washed with HBSS, and the number of branch points/tubes was quantified as described previously (36, 37).

**Statistical analysis**

The data were analyzed using two-tailed Student t tests for paired and unpaired samples. Values of p < 0.05 were considered significant.

**Results**

**Local expression of IL-17 in mouse ankles induces joint inflammation and vascularity**

Local expression of IL-17 using an adenoviral vector (10^7 PFU) resulted in increased inflammation, synovial lining thickness, and bone erosion in the ankles of C57BL/6 mice, compared with Ad-CMV–infected controls (10^7 PFU) (data not shown). The Ad-IL-17–treated group demonstrated significantly greater ankle circumference (data not shown) on days 4 and 10 postinjection compared with the control group. Von Willebrand factor staining of ankles harvested from day 10 postinjection demonstrated that Ad-IL-17–treated mice have significantly higher endothelial staining compared with the control group (Fig. 1). The concentration of joint IL-17 in the IL-17–induced arthritis model was 1200 and 400 pg/mg compared with 47 and 31 pg/mg detected in the Ad-CMV control group on days 4 and 10 post-Ad injection. These results suggest that IL-17 may be important for angiogenesis in vivo.

**IL-17 induces angiogenesis in vivo in Matrigel plugs**

The role of IL-17 on angiogenesis in vivo was assessed by determining its effect on blood vessel formation in Matrigel plugs in mice by using hemoglobin quantification, as well as Masson’s trichrome staining. The hemoglobin content of the IL-17–treated group was 10 times greater (p < 0.05) than the PBS control (Fig. 2A). Matrigel blood vessel formation was also examined histologically by using H&E (Fig. 2C, 2E, 2G) and Masson’s trichrome staining (Fig. 2D, 2F, 2H). The histological analysis demonstrated that IL-17 markedly enhances (p < 0.05) blood vessel growth compared with the control group (Fig. 2B). The levels of IL-17 detected in Matrigel plugs harvested on day 10 were 198 ± 35 pg/ml, which is within the range detected in IL-17–induced arthritis model as well as in the RA synovial fluid (mean was 233 pg/ml). These results support the role of IL-17 in angiogenesis in vivo.

**IL-17 induces endothelial (HMVEC) migration**

Experiments were performed to determine whether IL-17 is directly chemotactic for endothelial cells. For this purpose, chemotaxis was performed in a Boyden chamber with varying concentrations of IL-17, as well as positive (VEGF; 10 ng/ml) and negative (PBS) controls. IL-17 was chemotactic for HMVECs at concentrations ranging from 0.01 ng/ml (p < 0.05) to 100 ng/ml (p < 0.05) (n = 5) (Fig. 3A). The mean concentration of IL-17 in the 30 RA synovial fluids analyzed was 233 ± 64 pg/ml (21), a concentration that was highly chemotactic for HMVEC migration. IL-17’s endotoxin levels were quantified by using Limulus amebocyte cell lysate assay. At a concentration of IL-17 10-fold higher than that used in the HMVEC chemotaxis assay (500 ng/ml), the level of endotoxin was below the detection limit for this assay (<0.01 ng/ml LPS) (data not shown), suggesting that our results are specifically due to IL-17 and not endotoxin contamination. Supporting this interpretation, heat inactivation of IL-17 or incubation of IL-17 (50 ng/ml) with neutralizing Ab to IL-17 suppressed HMVEC migration (Fig. 3B).

We next determined the effect of IL-17 on chemokinesis. In the absence of IL-17 in the lower chamber, IL-17 in the upper chamber was chemotactic for HMVECs. When equivalent or higher concentrations of IL-17 were present in the lower compartment, no enhanced migration of HMVECs occurred (Fig. 3C). Taken together, our results suggest that IL-17 is chemotactic, not chemokinetic, for endothelial cells.

**IL-17 receptors are involved in IL-17–induced HMVEC chemotaxis and HMVEC tube formation**

Our results demonstrate that skin and lung HMVECs as well as HUVECs express significantly higher levels of IL-17RA and IL-17RC compared with HEK 293 cells (Fig. 4A, 4B). These experiments were performed to determine which IL-17 receptor is involved in HMVEC chemotaxis and tube formation. Although some reduction of HMVEC chemotaxis was noted with an anti–IL-17RA Ab, it was not significant. Inhibition of chemotaxis was significant when IL-17RC (40%) or both receptors (47%) were neutralized (Fig. 5A). IL-17 also induced HMVEC tube formation in Matrigel. Using concentrations of IL-17 ranging from 0.001 to 100 ng/ml, the optimal concentration of IL-17 to induce HMVEC tube formation was 50 ng/ml (p < 0.05) (data not shown). Consistent with the HMVEC chemotaxis data, neutralization of IL-17RC (30%) or both IL-17 receptors (45%) on HMVECs significantly decreased (p < 0.05) IL-17–mediated HMVEC tube formation (Fig. 5B–H). Blockade of IL-17RA alone was inefficient in this process. Collectively, our data suggest that IL-17RC plays a more important role in chemotaxis and tube formation.
in IL-17–mediated HMVEC chemotaxis and tube formation compared with IL-17RA.

**IL-17 activates ERK, JNK, and PI3K pathways in HMVECs; however only inhibition of PI3K reduces IL-17-induced HMVEC chemotaxis and tube formation**

To determine which signaling pathways in HMVECs are activated by IL-17, phosphorylation of the MAPK and AKT pathways was determined by Western blot analysis. Our data demonstrate that IL-17 phosphorylates ERK, JNK, and AKT1 as early as 15 min. Although the activation of ERK and JNK is gradually reduced at 75 and 65 min (Fig. 6B, 6C), respectively, AKT1 is still strongly phosphorylated at 75 min (Fig. 6A). The p38 pathway was not activated by IL-17 in HMVECs (data not shown). To determine which signaling pathways mediate HMVEC migration, chemical inhibitors at concentrations of 1 and 5 μM were used, whereas 10 μM was toxic and resulted in cell death, as determined by trypan blue staining (data not shown). Inhibition of ERK and JNK was ineffective in suppressing IL-17–induced HMVEC chemotaxis, whereas inhibition of PI3K reduced (p < 0.05) chemotaxis starting at 1 μM (Fig. 6D). Similarly, although inhibition of PI3K (starting at 1 μM) reduced IL-17–mediated tube formation by 30–40% (p < 0.05) (Fig. 7), suppression of ERK and JNK had no effect on this process. These results suggest that IL-17–induced HMVEC chemotaxis and tube formation are mediated through the PI3K/AKT1 pathway.

**IL-17 and its receptor are involved in RA synovial fluid-mediated HMVEC chemotaxis**

Next, we asked whether the IL-17 identified in human RA synovial fluid is chemotactic for HMVECs. In these experiments, we demonstrated that human RA synovial fluid is chemotactic for HMVECs, similar to positive control VEGF (Fig. 8A). Furthermore, human RA synovial fluid immunodepleted with anti–IL-17 significantly reduced HMVEC chemotaxis (p < 0.05), compared with control IgG-treated fluids. Neutralization of IL-17RA and RC on HMVECs showed that only IL-17RC was effective in suppressing human RA synovial fluid-mediated HMVEC migration (Fig. 8B). These results suggest that IL-17 and its receptor IL-17RC may play an important role in angiogenesis in RA. Furthermore, we demonstrate that immunodepletion of IL-17 and VEGF in RA synovial fluids does not have an additive or synergistic effect in reduction of HMVEC migration beyond the effect noted with neutralization of one factor alone (Fig. 8C), suggesting that both IL-17 and VEGF may be mediating HMVEC migration through the same signaling pathway.

**Discussion**

In this study, we show that IL-17 increases vascularity in experimental arthritis and induces blood vessel development in Matrigel plugs in vivo. Therefore, studies were performed to determine whether IL-17 might directly mediate angiogenesis and whether IL-17 contributes to neovascularization in RA. Our data demonstrate that IL-17 induces HMVEC chemotaxis at concentrations present in human RA synovial fluid. This effect is directly mediated by IL-17, because heat inactivation and neutralizing Abs to IL-17 and/or IL-17 receptors abrogate IL-17–induced HMVEC chemotaxis. We further demonstrate that IL-17–induced HMVEC chemotaxis and tube formation are mediated primarily through ligation to IL-17RC on HMVECs and activation of PI3K. We show that human RA synovial fluid-mediated HMVEC chemotaxis is markedly reduced by neutralization of IL-17 in the synovial fluids or blocking of IL-17RC on HMVECs. Last, we demonstrate that IL-17 and VEGF-immunodepleted RA synovial fluid does not reduce HMVEC chemotaxis any further than neutralization of each factor by itself.

In this paper, we confirm the results of others demonstrating that the local expression of IL-17 in mouse ankle joints induces arthritis (9). Histological analysis of mice receiving intra-articular injections of IL-17 demonstrated that IL-17 plays an important role in joint neutrophil migration (42). We demonstrate that IL-17–induced arthritis is associated with increased vascularity. Others have shown that IL-17 can promote tumor growth by upregulating proangiogenic factors such as VEGF and matrix metalloproteinase-9 from tumor cells, suggesting that IL-17 is indirectly associated with angiogenesis (43). We have also shown that forced ectopic expression of IL-17 induces expression of proangiogenic CXC (ELR+) chemokines in mouse ankles (S. Pickens and S. Shahrara, unpublished data).

On the basis of our results from the IL-17–induced arthritis model, we hypothesized that IL-17 may be important for angiogenesis in RA. Because there is some evidence demonstrating that

**FIGURE 2.** IL-17 enhances blood vessel growth in Matrigel plugs in vivo. A, IL-17 (2 μg) induced angiogenesis in the Matrigel plugs to a significantly greater degree compared with PBS control. Matrigel containing bFGF (20 ng/ml) served as positive control. The values represent the concentration of hemoglobin (grams per deciliter)/plug weight (grams) ± se, with n = 10. B, The histology was quantified on a score of 0–4 scale in Matrigel plugs that contained PBS, IL-17, or bFGF using H&E and Masson’s trichrome staining. A representative assay shows H&E (C, E, G) and Masson’s trichrome (D, F, H) staining of blood vessels in paraffin sections of Matrigel plugs containing PBS (C, D), IL-17 (E, F), and FGF (G, H) that was histologically scored in B (original magnification ×200). Values demonstrate mean ± SE, with n = 5. ∗p < 0.05.
IL-17 alone is unable to induce angiogenesis but can indirectly promote HMVEC chemotaxis by producing proangiogenic factors (29, 30) from RA synovial tissue fibroblasts, we investigated the role of IL-17 on HMVEC migration and tube formation. Our results demonstrate that IL-17 induces HMVEC chemotaxis at concentrations available in the human RA joint, which is mostly due to its ligation to IL-17RC. Although IL-17RC plays a major role in IL-17–mediated HMVEC chemotaxis and tube formation, neutralization of both receptors is more effective in this process compared with IL-17RC alone. Like monocytes, HMVECs express both IL-17RA and IL-17RC (21). However, in contrast to HMVEC chemotaxis, IL-17–mediated monocyte migration is induced through both IL-17RA and RC (21). Interestingly, a novel IL-17 receptor-like protein has been identified in HUVECs that interacts with FGF1 and inhibits activation of the ERK pathway and production of FGF, indicating that various IL-17 receptors may modulate angiogenesis differently (44).

Next, experiments were performed to investigate signaling pathways that were associated with IL-17–induced HMVEC chemotaxis and tube formation. Inhibition of the IL-17–activated pathways in HMVECs demonstrated that only activation of PI3K significantly reduces IL-17–mediated chemotaxis and tube formation, and suppression of ERK and JNK pathways was ineffective in this process. Consistently, CCL2/MCP-1–mediated endothelial chemotaxis is through activation of PI3K, as well as the ERK pathway (45). Furthermore, others have shown that PI3K signaling plays an important role in regulation of VEGF production as well as VEGF-mediated endothelial migration (46, 47), suggesting that PI3K is involved in the mediation of angiogenesis by various proinflammatory factors. A recently published article shows that increased expression of IL-17 in IFNγ−/− mice mediates tumor growth and angiogenesis through STAT3 phosphorylation (43). In contrast to IL-17, which promotes angiogenesis, IL-17F inhibits HUVEC tube formation, indicating that different IL-17 isoforms may modulate angiogenesis differently (48).

A recent publication demonstrated that tumor growth in s.c. and lung metastases are enhanced in IL-17−/− mice compared with the wild-type controls, suggesting that IL-17 may suppress tumor growth.
development (27). Conversely, others have shown that IL-17 markedly increases neovascularization in rat cornea (28) and vascularization in tumors (49), indicating that IL-17 may promote angiogenesis. However, there is also evidence demonstrating that IL-17 induces production of proangiogenic factors including NO, hepatocyte growth factor, CXCL1/KC, CXCL2/MIP-2, PGE,

**FIGURE 5.** IL-17–mediated HMVEC chemotaxis and tube formation are regulated through both IL-17RA and IL-17RC. A, HMVECs were incubated with mouse anti-human IL-17RA and IL-17 RC Abs (10 μg/ml) or control IgG (10 μg/ml) for 1 h. Thereafter, HMVEC chemotaxis was performed in response to IL-17 (50 ng/ml) for 2 h. PBS was used as a negative control and VEGF (60 nM) as a positive control. HMVECs were incubated with Abs to IL-17RA, IL-17RC, both IL-17RA and RC, or IgG for 45 min at 37˚C. Cells were then added to polymerized Matrigel, IL-17 (50 ng/ml), placed in the wells, and the plate was incubated for 16 h at 37˚C (in triplicate). Photomicrographs (original magnification ×100) taken of representative wells treated with PBS (B), FGF (20 ng/ml) (C), IL-17 (50 ng/ml) plus IgG (D), IL-17 (50 ng/ml) plus anti–IL-17RA (10 μg/ml) (E), IL-17 (50 ng/ml) plus anti–IL-17RC (10 μg/ml) (F), and IL-17 (50 ng/ml) plus anti–IL-17RA and RC (10 μg/ml) (G) in which IL-17–induced tube formation is significantly reduced by the neutralization of IL-17RC or both receptors (p < 0.05). H, Data presented demonstrates mean number of branch points/tubes in each treatment group. Values are the mean ± SE, n = 3. *p < 0.05.

**FIGURE 6.** IL-17–induced HMVEC migration is suppressed by PI3K inhibition. To determine the mechanism of IL-17 in HMVECs, cells were stimulated with IL-17 (50 ng/ml) for 0–75 min, and the cell lysates were probed for p-AKT (A), pERK (B), or pJNK (C). These results are representative of three experiments. D, To determine signaling pathways associated with IL-17–induced HMVEC migration, cells were treated with the identified chemical inhibitors for PI3K (LY294002; 1 and 5 μM) or ERK (PD98059; 1 and 5 μM) as well as JNK (SP600125; 1, 5 μM) 2 h in the Boyden chamber. Only inhibition of PI3K downregulated IL-17–induced HMVEC migration. Values demonstrate mean ± SE of three experiments in triplicate. *p < 0.05.
PGE2, and VEGF from RA synovial fibroblasts, and the production of some of these factors is further enhanced by TNF-α (50). Therefore, we investigated the contribution of IL-17 to human RA synovial fluid-mediated HMVEC chemotaxis. Neutralization of IL-17 in RA synovial fluid partially reduced RA synovial fluid-mediated HMVEC chemotaxis. RA synovial fluid-mediated HMVEC chemotaxis was mediated through IL-17RC, confirming the importance of this receptor in IL-17–mediated angiogenesis. Interestingly, angiogenic factors present in human RA synovial fluid are mostly produced by RA synovial tissue fibroblasts (VEGF, bFGF, VCAM1, IL-6, and ELR+ CXC chemokines) or macrophages (TNF-α, IL-8, and IL-1β) (25). IL-17 is the only

FIGURE 7. IL-17–mediated tube formation is reduced by inhibition of PI3K. HMVECs were incubated with inhibitors to PI3K (LY294002; 1 and 5 μM), ERK (PD98059; 1 and 5 μM), INK (SP600125; 1 and 5 μM), or DMSO for 45 min at 37˚C prior adding to polymerized Matrigel. IL-17 (50 ng/ml) was then added to the wells, and the plate was incubated for 16 h at 37˚C (in triplicate). Photomicrographs (original magnification ×100) taken of representative wells treated with PBS (A), FGF (20 ng/ml) (B), IL-17 (50 ng/ml) plus DMSO (C), IL-17 (50 ng/ml) plus LY294002 (5 μM) (D), IL-17 (50 ng/ml) plus PD98059 (5 μM) (E), and IL-17 (50 ng/ml) plus SP600125 (5 μM) (F) in which IL-17–induced tube formation is significantly reduced by the inhibition of PI3K/AKT1 pathway (p < 0.05). G demonstrates mean number of tubes per well where LY294002 (1 and 5 μM; PI3K/AKT1 inhibitor) significantly reduces the number of branch points induced by IL-17 activation in Matrigel tube formation assay, whereas ERK (PD98059; 1 and 5 μM) and JNK inhibitors (SP600125; 1 and 5 μM) were ineffective. Values are the mean ± SE, n = 3. *p < 0.05.

FIGURE 8. RA synovial fluid-induced HMVEC chemotaxis is mediated by IL-17 through ligation to IL-17RC, and IL-17 does not synergize with VEGF in RA synovial fluid-induced HMVEC migration. A, Anti–IL-17 (10 μg/ml) or control IgG was added to RA synovial fluids from eight patients (1/20 dilution) (1 h at 37˚C) prior to performing HMVEC chemotaxis in response to human RA synovial fluids. B, HMVECs were incubated with Abs to IL-17 RA and RC (10 μg/ml), as well as isotype control for 1 h prior to performing HMVEC chemotaxis in response to eight human RA synovial fluids. C, RA synovial fluids from eight patients (1/20 dilution) were incubated with Abs to IL-17 (10 μg/ml), VEGF (10 μg/ml), or both as well as isotype control or PBS or VEGF for 1 h prior to performing HMVEC chemotaxis in response to RA synovial fluids. The values represent the mean ± SE. *p < 0.05.
lymphokine that contributes to human RA synovial fluid-mediated angiogenesis, suggesting that T cells may also be important in this process. The data presented in this study, together with our previously reported evidence demonstrating that IL-17 is important for monocyte migration (21) in RA synovial fluids, highlights the importance of IL-17 in RA pathogenesis.

Interestingly, our results show that neutralization of IL-17 and VEGF do not synergize in reducing RA synovial fluid-induced HMVEC migration beyond the effect detected with one factor alone. As shown with IL-17, VEGF-induced HMVEC chemotaxis is mediated through PI3K (46, 51). Therefore, the lack of synergy between IL-17 and VEGF in inducing HMVEC chemotaxis may be due to that both mediators are using the same signaling pathway or that as shown previously HMVEC cell migration is within a bell shaped curve (37, 52); therefore, the synergistic effect could not be detected.

In conclusion, endothelial migration and tube formation induced by IL-17 were mediated through activation of the PI3K pathway and ligation to both IL-17 receptors. However, neutralization of only IL-17 or IL-17RC significantly downregulated human RA synovial fluid-mediated endothelial migration, suggesting that IL-17 plays an important role in RA angiogenesis.

**Disclosures**

The authors have no financial conflicts of interest.

**References**

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