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Evidence of IL-18 as a Novel Angiogenic Mediator

Christy C. Park, Jacques C. M. Morel, M. Asif Amin, Matthew A. Connors, Lisa A. Harlow, and Alisa E. Koch

Angiogenesis, or new blood vessel growth, is a key process in the development of synovial inflammation in rheumatoid arthritis (RA). Integral to this pathologic proliferation are proinflammatory cytokines. We hypothesized a role for IL-18 as an angiogenic mediator in RA. We examined the effect of human IL-18 on human microvascular endothelial cell (HMVEC) migration. IL-18 induced HMVEC migration at 1 nM (p < 0.05). RA synovial fluids potently induced endothelial cell migration, but IL-18 immunodepletion resulted in a 68±5% decrease in HMVEC migration (p < 0.05). IL-18 appears to act on HMVECs via αβ integrin. To test whether IL-18 induced endothelial cell tube formation in vitro, we quantitated the degree of tube formation on Matrigel matrix. IL-18, 1 or 10 nM, resulted in a 77% or 87% increase in tube formation compared with control (p < 0.05). To determine whether IL-18 may be angiogenic in vivo, we implanted IL-18 in Matrigel plugs in mice, and IL-18 at 1 and 10 nM induced angiogenesis (p < 0.05). The angiogenesis observed appears to be independent of the contribution of local TNF-α, as evidenced by adding neutralizing anti-TNF-α Ab to the Matrigel plugs. In an alternative in vivo model, sponges embedded with IL-18 or control were implanted into mice. IL-18 (10 nM) induced a 4-fold increase in angiogenesis vs the control (p < 0.05). These findings support a novel function for IL-18 as an angiogenic factor in RA and may elucidate a potential therapeutic target for angiogenesis-directed diseases.


IL-18 is a cytokine with many proinflammatory functions. IL-18 was initially described as IFN-γ-inducing factor in 1989 (15). Thus, IL-18 stimulates Th1 cytokines by T cells and NK cells and promotes Th1 cell differentiation and immune responses (16, 17). IL-18 can act in synergy with IL-12 in regulating IFN-γ (18). However, IL-18 also up-regulates other cytokines, such as TNF-α, IL-1β, and IL-8 in non-CD14+ PBMCs, resting T cells, and NK cells (19). IL-18 up-regulates GM-CSF in PBMCs (20) and also in synovial membrane cultures, directly affecting macrophages to stimulate cytokine production (21).

IL-18 has structural homology with IL-1, shares some common signaling pathways (22), and also requires the cleavage at its aspartic acid residue by IL-1-converting enzyme to become the active, mature protein (23, 24). Thus, IL-1 and IL-18 share many biologically similar inflammatory functions. Previous work has implicated IL-18 in RA, as higher levels are present in RA vs osteoarthritic synovial fluid (SF) and sera (21). Also, IL-18 enhanced erosive, inflammatory arthritis in a murine model of systemic arthritis (21). In the study by Leung et al. (25) in which IL-18 promoted collagen-induced inflammatory arthritis through a mechanism shown to be distinct from IL-12, cytokine treatment enhanced synovial hyperplasia, inflammatory infiltrate, and cartilage erosion. Interestingly, the IL-18-treated mice produced significant amounts of TNF-α, and splenic macrophages from mice cultured with IL-18 also stimulated high levels of TNF-α. The for IL-18 in articular inflammation was confirmed in mice lacking the IL-18 gene that had reduced incidence and severity of collagen-induced arthritis, which was reversed by treatment with rIL-18 (26). One important source of IL-18 is the macrophage, which is a critical synovial tissue producer of IL-12 (27), and responsible for the production of many other cytokines (28).

However, various other sources of IL-18 have been identified, including Kupffer cells, dendritic cells, keratinocytes, articular chondrocytes, osteoblasts, and synovial fibroblasts (18, 21, 29–33). In regard to arthritis, if IL-18 functions in an autocrine or paracrine fashion, the increased expression of IL-18 in the synovium...
may play a critical role in the development of synovial inflammation, synovial hyperplasia, and articular degradation, to which angiogenesis may contribute. Given the importance of angiogenesis in the pathophysiology of RA, we hypothesized a role for IL-18 as an angiogenic mediator. Supportive of this function is the finding that IL-18 has been shown to stimulate production of angiogenic TNF-α (19). The establishment of the role of IL-18 in inflammation and RA led to the question of its angiogenic potential.

In this study, we examined the capacity of IL-18 to mediate angiogenesis in vitro and in vivo models. We further compared the angiogenic activity of IL-18 with that of known mediators, such as bFGF. One potential mechanism of the angiogenic activity of IL-18 that we examined is through the involvement of αβ3 integrin (34). This role of αβ3 was examined in the IL-18-induced migration of endothelial cells. IL-18 induced tube formation in Matrigel matrix in vitro. To examine the role of IL-18 in angiogenesis in vivo, we also implanted IL-18 in Matrigel plugs in mice, and found a significant increase in blood vessel formation over control, as measured by hemoglobin concentration. This effect was not decreased by implanting neutralizing anti-TNF-α Ab in the Matrigel plug. Finally, in an alternative model of angiogenesis, a wound granuloma model, we implanted sponge discs embedded with IL-18 into mice and showed a 4-fold increase in angiogenesis compared with controls. These findings support a novel role for IL-18 as an angiogenic mediator in RA and may elucidate a potential therapeutic target for angiogenesis-directed disease.

Materials and Methods

Reagents

Recombinant human (rh) IL-1β and TNF-α, with sp. act. of 2 × 10^7 and 1.3 × 10^7 U/mg, respectively, were gifts from Upjohn-Pharmacia (Kalamazoo, MI). Some TNF-α was from R&D Systems (Minneapolis, MN). rhIL-18 was purchased from R&D Systems, with sp. act. measured by ability to induce IFN-γ production by KG-1 cells in the presence of 10 ng/ml rhTNF-α (100 ng/ml rhIL-18 induces 0.5–2 ng/ml IFN-γ produced by 10^6/ml KG-1 cells), and was used for the majority of the assays. An additional source of rhIL-18 was PeproTech (Rocky Hill, NJ), with IL-18 sp. act. determined by the dose-dependent stimulation of IFN-γ production by human PBMC costimulated with human IL-12 (ED50 of 5 ng/ml); and the experiments in which this IL-18 was used included the checkerboard assay, the chemotaxis assays comparing the activity of IL-18 with IL-8 or the experiments in which this IL-18 was used included the checkerboard analysis.

In this study, we examined the capacity of IL-18 to mediate angiogenesis in vitro and in vivo models. We further compared the angiogenic activity of IL-18 with that of known mediators, such as bFGF. One potential mechanism of the angiogenic activity of IL-18 that we examined is through the involvement of αβ3 integrin (34). This role of αβ3 was examined in the IL-18-induced migration of endothelial cells. IL-18 induced tube formation in Matrigel matrix in vitro. To examine the role of IL-18 in angiogenesis in vivo, we also implanted IL-18 in Matrigel plugs in mice, and found a significant increase in blood vessel formation over control, as measured by hemoglobin concentration. This effect was not decreased by implanting neutralizing anti-TNF-α Ab in the Matrigel plug. Finally, in an alternative model of angiogenesis, a wound granuloma model, we implanted sponge discs embedded with IL-18 into mice and showed a 4-fold increase in angiogenesis compared with controls. These findings support a novel role for IL-18 as an angiogenic mediator in RA and may elucidate a potential therapeutic target for angiogenesis-directed disease.

Bioassay for HMVEC migration activity

Subconfluent HMVECs (passage 3–12) were fed the night before the assay, to optimize the cellular conditions, with endothelial cell basal medium-2 (EBM-2; Clonetics) and 0.1% FBS. HMVECs (3.75 × 10^5 cells/25 µl EBM and 0.1% FBS) were placed in the bottom wells of a 48-well Boyden chemotaxis chamber (NeuroProbe, Cabin John, MD) with gelatin-coated polycarbonate membranes. Nonspecific antibody (NSAb) was added to the bottom well. The plates were inverted and incubated in a humidified incubator at 37°C under 5% CO2/95% air for 2 h, allowing endothelial cell attachment to the membrane. The chambers were reinserted, and the test substances, PBS, positive control bFGF (60 nM) in PBS, or RA SF were added to the wells in the top of the chamber, and the chamber was further incubated for 2 h at 37°C. IL-18 (R&D Systems) was used in varying concentrations (10 nM, 0.025 nM, 0.05 nM, and 0.125 nM) in PBS, and PBS with 2% BSA, the diluent used to reconstitute the lyophilized cytokines, resulted in similar baseline levels of HMVEC migration. Additionally, this assay was also performed with a representative RA SF sample after incubation for 1 h at 37°C with combinations of neutralizing Ab (25 µg/ml) to human IL-18 and ENA-78 or IL-8 before adding samples to the bottom of the wells. Comparison was made to control nonspecific Abs, goat IgG for anti-IL-18 control, and mouse IgG for anti-IL-8 and anti-ENA-78. A confirmatory assay was performed with rhIL-18 and the addition of neutralizing anti-human IL-18 Ab or nonspecific isotype-matched control Ab (25 µg/ml of each). A comparative dose response to the cytokines IL-18, IL-8, and ENA-78 (PeproTech) was also examined. The membranes were then re-incubated for 1 h in methanol (100%), and stained with Diff-Quik. Readings represent the number of cells migrating through the membrane (the sum of three high power ×40 fields/well, averaged for each quadruplicate well).

Checkerboard analysis

Checkerboard analyses were performed as previously described (14, 35) using the Boyden chamber. Briefly, increasing concentrations of IL-18 (0, 1, 10, and 100 nM) were added to the cell suspensions in the bottom wells in addition to the top wells. The effect of an alteration or decrease in the resultant gradient was examined. The assay was performed three times and analyzed in a similar manner as above.

The role of αβ3 integrin in IL-18-mediated migration

To determine whether IL-18 induces endothelial migration via αβ3 integrin, we employed the modified Boyden chamber migration assay. HMVECs were preincubated for 1 h at 37°C with Ab to integrin αβ3 (25 µg/ml), in an attempt to inhibit its function in the migration process, or nonspecific isotype-matched control Ab. The cell suspensions were then resuspended in fresh medium again with Ab or αβ3 or nonspecific Ab and used in the cell migration assay, as described above. The test substances included IL-18 (10 nM), bFGF (60 nM) as positive control, and PBS as negative control. This assay was performed three times, with additional positive control TNF-α (15 ng/ml) or negative control VEGF (10 ng/ml).

IL-18 induced HMVEC proliferation

HMVEC proliferation in response to rhIL-18 was determined using a modified method from previously described procedures (14, 36). Briefly, HMVECs fed 1 day before use (with a fresh media exchange to obtain optimal growth conditions) were trypsinized and resuspended at 5 × 10^4 cells/ml in EBM (Clonetics) plus 2% FBS + gentamicin. An equivalent number of cells (2.5 × 10^3) in 50 µl cell suspension was plated nonconfluently onto each well of a 96-well plate and allowed to adhere for 4 h at 37°C. Serial IL-18 dilutions were prepared in this media at 2× concentration, with 50 µl/well; the 50 µl/well was examined. The 50 µl was serially diluted in the wells, resulting in 100 µl 1× dilutions. In a similar manner, serial dilutions of bFGF were also prepared for a positive control; media alone were added for the negative control. All dilutions were performed in quadruplicate wells. The plate was incubated for up to 72 h at 37°C. The Cell Titer 96 Aqueous nonradioactive cell proliferation assay kit (Promega, Madison, WI), which contains a tetrazolium compound and phenazine methosulfate solution, was diluted according to the recommended protocol and added to each well (20 µl/well). The plates were incubated at 37°C for 2–12 h until color development occurred. Absorbance was read at 490 nm on an ELISA plate reader with Microplate Manager (Bio-Rad, Richmond, CA). The absorbance readings were performed when the maximal response (plateau value) of bFGF concentrations represented at least a doubling from baseline control (no stimulation), and the absorbance at 490 nm (y-axis) vs concentration of stimulant (x-axis) was plotted and examined. Proliferation curves for HMVECs were determined, and measurements were obtained and compared in the log proliferation phase, which was 3 days (r = 72 h).
for the conditions described. Serial dilutions of IL-18, ranging from 1 nM to 100 nM, or bFGF (positive control), ranging from 10 nM to 100 nM, in EBM and 2% FBS in a 100-μl vol were assayed.

**Matrigel in vitro HMVEC tube formation assay for in vitro angiogenesis**

Matrigel was used to examine HMVEC tube formation in response to IL-18. Matrigel was plated in eight-well chamber slides after thawing on ice, and the Matrigel was allowed to polymerize at 37°C for 30–60 min. HMVECs were removed from culture, trypsinized, and resuspended at 4 x 10^4 cells/ml in Medium 199 (Invitrogen, Carlsbad, CA) containing 2% FBS and 200 μg/ml endothelial cell growth supplement (Becton Dickinson, Bedford, MA). Four hundred microliters of cell suspension were added to each chamber, followed by 1, 5, or 10 nM IL-18, 50 nM PMA, or vehicle control (PBS for IL-18, and DMEM for PMA) placed directly into the suspension in the chambers. The chamber slides were then incubated for 16–18 h at 37°C in 5% CO2 humidified atmosphere. Culture media were aspirated off the Matrigel surface, and the cells were fixed with methanol and stained with Diff-Quick Solution II. Each chamber was photographed using Polaroid Microcam camera at ×22 magnification. The number of tubes formed was quantitated blindly, as previously described (36).

Briefly, a connecting branch between two discrete endothelial cells was counted as one tube and required a consistent intensity, thickness, and minimum length (>1 mm on a 4× enlarged copy of the photomicrograph) to be counted. This tube analysis was determined from one focal plane graphed using Polaroid Microcam camera at ×22 magnification. The number of tubes formed was quantitated blindly, as previously described (36).

**Masson’s trichrome staining of Matrigel plugs**

To examine the Matrigel plugs histologically, some of the plugs were paraffin embedded. Sections (5 μm) were deparaffinized and stained using Masson’s trichrome staining. In brief, sections were hydrated in distilled water, dipped in Bouin’s solution for 1 h at 56°C, washed in water, dipped in Weigert’s iron solution for 7 min, washed, and dipped in Biebrich scarlet-acid fuchsin solution for 2 min. Sections were then rinsed, incubated in phosphomolybdic-phosphotungstic acid solution for 10 min, dipped in aniline blue solution for 5 min, rinsed, dipped in glacial acetic acid solution for 5–10 min, and dehydrated in two changes of 95% alcohol, 100% alcohol, and xylene. The slides were mounted with Cytoseal 60 (Stephens Scientific, Kalamazoo, MI).

**Sponge granuloma in vivo angiogenesis assay**

The animal model of inflammatory angiogenesis using a sponge granuloma has been described by Fajardo and colleagues (41). C57BL/6 mice were divided into several groups: negative control PBS; positive control, αFGF; and test substance, IL-18. One-centimeter sponge discs cut from sheets of 2-mm-thick polystyrene sponge foams obtained from sterile packs (M-pact, Eudora, KS) were prepared, and a 2-mm hole was cut into the disc center to serve as a depot for control PBS or test substances, and then coated back with the cut plug. After adding the stimulant to the center hole, the sponge discs were coated with an inert slow release ethylene vinyl acetate copolymer, Elvax (DuPont Packages), and both disc surfaces were sealed with Millipore filters (0.45 μm) using Millipore glue number 1 (Millipore, Bedford, MA). The animals were anesthetized with pentobarbital 40 mg/kg of body weight, the hair on their left back area was shaved and sprayed with 70% ethanol, and the disc inserted into the s.c. layer at a site 2 cm distant from the incision, which was then sutured to prevent disc dispersion. After 9–12 days, the animals were sacrificed, and the sponge discs were harvested. The sponges were then analyzed by hemoglobin quantitation.

**Hemoglobin quantitation of sponge granulomas by tetramethylbenzidine**

To quantitate angiogenesis in the sponge model, the sponges were placed in 2 ml double-distilled water in 24-well plates. The sponges were cut into small pieces with scissors and then homogenized with a Kontes’ homogenizer. The samples were then spun at 8000 rpm for 6 min, and the resultant supernatants were filtered through a 0.22-μm filter. The Drabkin’s reagent 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 after 3–5 min. Absorbance was read with an ELISA plate reader at 450 nm. To calculate hemoglobin concentrations, the values were normalized by the weight of the sponges.

**Statistical analysis**

Statistical analysis was performed using the nonparametric Wilcoxon rank-sum test, with statistical significance defined by p < 0.05. Values were expressed as means ± SEM.

**Results**

**IL-18 induces HMVEC migration**

To determine the effect of rhIL-18 on HMVEC migration, we tested varying concentrations of IL-18 in a modified Boyden chamber assay. A representative assay of three is shown (Fig. 1). IL-18 induced HMVEC migration in a concentration-dependent manner, reaching significance at 1 nM (p < 0.05). Cell migration measured by the number of migrating cells/well was determined by three blinded readings per each quadruplicate well. The mean cell migration induced by 1 and 10 nM IL-18 was 18 ± 0.8 and 20 ± 0.4 cells/well, respectively, while 100 nM IL-18 (29 ± 3.3 cells/well) exceeded cell migration by control stimulant 60 nM bFGF (24.7 ± 1 cells/well).
Neutralization of IL-18 inhibits HMVEC migration

To confirm the specificity of rhIL-18-induced HMVEC migration, we preincubated the IL-18 stimulant with either neutralizing goat anti-human IL-18 IgG Ab or nonspecific isotype-matched control IgG Ab, at a concentration of 25 μg/ml for 1 h before and during the 2 h of the cell migration assay. The response to the stimulant with or without Ab was determined by the number of migrating cells/well based on three blinded readings per each quadruplicate well (Fig. 2A). Neutralization of IL-18 resulted in inhibition of cell migration to basal values (9 ± 1.32 cells/well) similar to that seen upon incubation of HMVECs with control PBS (7 ± 0.71 cells/well). Results are representative of three similar assays. In addition, 10 nM IL-18 alone stimulated HMVEC migration (27.25 ± 1.44 cells/well) comparable to that by 60 nM bFGF (28.75 ± 0.65 cells/well).

IL-18 is primarily chemotactic for HMVECs

The checkerboard analysis was performed with the chemotaxis assay using increasing concentrations of IL-18 in the cell suspensions at the bottom of the chamber as well as in the top of the chamber. A representative assay of three is shown (Fig. 2B). The concentrations were chosen for their known induction of HMVEC migration, starting at 1 nM, although the effect of 100 nM may not have been greatly dampened by the addition of 1 or 10 nM on the opposite side of the membrane. There was evidence of some HMVEC migration induced by the presence of IL-18 within the cell suspension, which exceeded that by PBS, reflecting a chemokinetic contribution. However, the presence of equivalent concentrations of IL-18 on either side of the membrane reduced the resultant migration to levels closer to PBS control. These data suggest a primarily chemotactic effect of IL-18 on HMVEC migration.

IL-18 contributes to RA SF-induced HMVEC migration

To determine the relative contribution of IL-18 to RA SF-induced HMVEC migration, we preincubated RA SF samples with neutralizing goat anti-human IL-18 Ab or nonspecific isotype-matched control goat IgG Ab, at a concentration of 25 μg/ml for 1 h before and during 2 h of the assay. We found significant inhibition of RA SF-induced HMVEC migration with anti-IL-18 Ab, compared with nonspecific Ab (Fig. 3). The inhibition of HMVEC migration after neutralization of the RA SF samples (n = 4) with the anti-IL-18 Ab (13.5 ± 2.9 cells/well) was 68 ± 5% compared with sham neutralization with the control IgG Ab (41.5 ± 4.3 cells/well). HMVEC migration with control IgG was equivalent to RA SF alone (40.6 ± 1.4 cells/well) (p < 0.05). These findings suggest a prominent role for IL-18 in RA SF-induced HMVEC migration.

Contribution of IL-18, IL-8, and ENA-78 to RA SF-induced HMVEC migration

To compare the relative contribution to RA SF-induced HMVEC migration, we preincubated four different RA SF samples with neutralizing Abs to IL-18, IL-8, or ENA-78 (Fig. 4). Immunodepletion of IL-18 resulted in a similar percentage of HMVEC migration inhibition (68 ± 5%) as immunodepletion of IL-8 (64 ± 7%). However, immunodepletion of IL-18 resulted in a 1.6-fold greater percent inhibition than immunodepletion of ENA-78 (43 ± 6%). Values shown are means generated from four different RA SF patient samples and are significant compared with isotype-matched control Ab (p < 0.05). Inhibition was not further enhanced with combinations of neutralizing Abs to IL-18 and either IL-8 or ENA-78 (62 ± 3% and 70 ± 3%, respectively).

Comparison of IL-18-induced HMVEC migration with chemokine-induced migration by IL-8 and ENA-78

The neutralization of IL-18 present in RA SF reduced SF-induced HMVEC migration to a similar or greater degree as neutralization of IL-8 or ENA-78, respectively.
these recombinant human cytokines in the HMVEC migration assay. The dose responses were generated from three different experiments, and a representative assay is shown (Fig. 5). IL-18 and ENA-78 induced HMVEC migration to a similar degree at the lower concentrations 0.1–10 nM. IL-18 exceeded the effect of ENA-78 at higher concentrations (10–100 nM). In comparison, IL-8 showed greater effect on HMVEC migration at the lower and higher doses compared with IL-18 and ENA-78, which had more comparable bioactivities. Overall, assaying a wide range of concentrations, it appears that IL-8 is a more potent angiogenic agent than IL-18, which is of greater potency than ENA-78.

**IL-18 induces HMVEC migration via αβ3 integrin**

To determine the mechanism by which IL-18 mediates angiogenesis, we hypothesized that IL-18 may act through integrins present on HMVECs. TNF-α is angiogenic in part via its action on integrin αβ3 (34). Thus, we examined whether blocking αβ3 would inhibit IL-18-induced angiogenesis in the HMVEC migration assay. HMVECs were first incubated with anti-αβ3 Ab or nonspecific control IgG at 25 μg/ml for 1 h before and during the chemotaxis assay. IL-18 (10 nM) was then used to stimulate HMVEC migration, and comparison was made with the control stimulants bFGF and TNF-α. To illustrate a contrasting mechanism, VEGF was also used as another stimulant, which would not be expected to be inhibited by blocking αβ3, since the angiogenic ability of VEGF is thought to occur via the integrin αβ3 (42). Blocking HMVEC αβ3 resulted in significant inhibition of IL-18-induced cell migration comparable with that seen with control (Fig. 6). The same effect was seen with 25 or 10 μg/ml blocking Ab concentration. Significant inhibition of cell migration by blocking HMVEC αβ3 before stimulation with bFGF and TNF-α was also demonstrated, while no change in cell migration occurred after stimulation with VEGF. Shown are the representative data from three separate assays.

**Effect of IL-18 on endothelial cell proliferation**

The effect of rhIL-18 on endothelial cell proliferation was assessed using varying concentrations of IL-18 (10−6 to 100 nM) to stimulate HMVEC growth. Control stimulation with bFGF (10−5 to 10 nM) demonstrated an expected 2-fold increase in HMVEC proliferation. However, IL-18 did not directly stimulate or inhibit HMVEC proliferation on repeated assays (n = 4), but behaved similarly to PBS control. The time point of 72 h at which cell growth was measured was selected when control bFGF-induced proliferation was optimal. Additionally, proliferation curves for HMVECs were determined for bFGF, media alone, and IL-18, and measurements were compared in the log proliferation phase (Fig. 7).

**IL-18 induces angiogenesis in Matrigel in vitro**

Endothelial cell tube formation in Matrigel is one measure of angiogenesis in vitro. The role of IL-18 in angiogenesis was assessed by determining the induction of HMVEC tube formation on Matrigel matrix plated onto eight-well chamber slides. Tube-like structures formed after a 16-h incubation in the presence of IL-18, whereas grossly fewer tubes formed with PBS control (Fig. 8).
Shown are representative wells (magnified $\times 88$): A shows tube formation with IL-18 (1 nM), compared with its PBS control (B), and C shows tube formation with IL-18 (10 nM), compared with its PBS control (D). IL-18 at 1 and 10 nM induced significant HMVEC tube formation with an increase of 77% and 87% compared with PBS control ($p < 0.05$). Results represent the average of three wells per four similar assays at two different IL-18 concentrations, in which the number of tubes formed per each well were blindly counted from photomicrographs taken by Polaroid at $\times 22$ magnification of the center of the well focused on the Matrigel surface. Shown are the data for 1 nM IL-18 stimulation (Fig. 8E). Comparison with the stimulant PMA and its vehicle control DMSO is also shown.

**IL-18 induces angiogenesis in Matrigel in vivo**

The angiogenic role of IL-18 in vivo was assessed by examining the effect of IL-18 on blood vessel growth in the Matrigel plugs in mice. The Matrigel plugs containing IL-18 (10 nM) induced significantly greater angiogenesis than control PBS. Histology illustrates the difference qualitatively in the representative photomicrographs of Masson trichrome-stained sections ($\times 50$ magnification) (Fig. 9A). Marked new blood vessel growth can be seen in the IL-18-containing plugs (left panel) compared with the control (right panel). Five Matrigel plugs were embedded with stimulus for each test group. The hemoglobin content of the 10 nM and 1 nM IL-18-containing plugs was 52-fold and 64-fold higher, respectively, than that for PBS control (Fig. 9B): $1.65 \pm 0.45$ g/dl/(mg) for 10 nM IL-18, $2.05 \pm 1.3$ g/dl/(mg) for 1 nM IL-18, and $0.032 \pm 0.02$ g/dl/(mg) for PBS ($p < 0.05$). IL-18-stimulated angiogenesis is comparable with positive control aFGF in the Matrigel plugs.

**FIGURE 6.** Anti-$\alpha_v\beta_3$ Ab inhibits IL-18-induced HMVEC migration. Inhibition of IL-18-induced HMVEC migration was demonstrated by preincubation of HMVECs with anti-$\alpha_v\beta_3$ Ab vs nonspecific control Ab. The effect of blocking $\alpha_v\beta_3$, integrin before stimulation with IL-18 or the positive controls bFGF and TNF-$\alpha$ is compared with nonspecific blockade with goat IgG. Results represent the mean number of cells/well $\pm$ SEM migrating, and shown is a representative assay ($n = 3$ assays). There was significant suppression of IL-18-induced HMVEC migration after $\alpha_v\beta_3$ blockade, as was seen with bFGF and TNF-$\alpha$. VEGF served as a negative control stimulant. Ab concentrations were 25 $\mu$g/ml.

**FIGURE 7.** IL-18 does not affect HMVEC proliferation. Varying concentrations of rhIL-18 ($10^{-6}$–100 nM) were used to assess the effect on HMVEC proliferation, which was compared with that of control bFGF ($10^{-3}$–10 nM) and nonstimulated PBS controls. Cell growth was measured as absorbance readings at 72 h, during the optimal phase of growth. An expected 2-fold increase was demonstrated after 100 pM and 1 nM bFGF stimulation, with significant difference from PBS controls seen also at 10 nM bFGF (*, $p < 0.05$). However, the effect of IL-18 was similar to nonstimulated PBS controls on repeated assays, and shown is a composite of the data ($n = 3$).

**FIGURE 8.** IL-18 induces endothelial cell tube formation on Matrigel in vitro. Photomicrographs (taken at $\times 22$, enlarged to $\times 88$ magnification) of representative wells show the increased IL-18-induced HMVEC tube formation in comparison with PBS control: A, IL-18 (1 nM) with B, PBS control; and C, IL-18 (10 nM) with D, PBS control. Shown are results representative of three experiments. E, HMVECs form significantly greater number of tubes in Matrigel matrix in response to 1 nM IL-18 vs PBS control (*, $p < 0.05$). Positive control stimulant is PMA; vehicle control is DMSO; and the control for IL-18 is PBS.
IL-18 induces angiogenesis in Matrigel in vivo independently of TNF-α

To examine the angiogenic effect of IL-18 in the presence of neutralizing anti-TNF-α Ab, Matrigel plugs containing 10 nM IL-18 and 25 μg/ml anti-TNF-α Ab or isotype-matched control IgG were injected into mice (n = 18 per group). After 7 days incubation, the mean normalized hemoglobin contents were determined, and there was no significant difference between the two groups: 2.67 ± 0.62 g/dl/(mg) for the IL-18 plus IgG-containing plugs vs 2 ± 0.31 g/dl/(mg) for the IL-18 plus anti-TNF-α-containing plugs (Fig. 10). The hemoglobin content of the 10 nM IL-18 plus IgG-containing plugs was 3.7 times greater than that of PBS control.

IL-18 induces angiogenesis in the sponge granuloma model in vivo

To examine whether IL-18 induced inflammatory angiogenesis in vivo, the sponge granuloma model was used. In this model, angiogenesis was measured as increased blood vessel growth in discs made from polyvinyl alcohol foam sponges containing either IL-18 or control stimulant aFGF and implanted into mice. After 11 days, there was a significant increase in blood vessel growth with IL-18 stimulation vs PBS control (Fig. 11). A 5-fold increase in angiogenesis with IL-18 (10 nM) was demonstrated by greater hemoglobin concentrations measured with tetramethylbenzidine, and the hemoglobin value induced by IL-18 was even greater than that for aFGF, which resulted in a 4-fold increase: 5.77 ± 1.21 g/dl/(mg) for IL-18, 4.45 ± 1.81 g/dl/(mg) for aFGF, and 1.13 ± 0.29 g/dl/(mg) for PBS (p < 0.05).

Discussion

In this study, we examined the potential role of IL-18 in mediating angiogenesis through its action in both in vitro and in vivo bioassays. We found evidence that IL-18 possesses angiogenic properties, a novel function for IL-18. While IL-18 has been implicated in RA pathogenesis, IL-18’s complex array of functions and mechanisms by which it promotes inflammation has not been fully elucidated. IL-18 is up-regulated in the SF and serum from RA patients as compared with osteoarthritis patients (21). The use of IL-18 has also been shown to facilitate the development of erosive, inflammatory arthritis when administered in murine type II collagen-induced arthritis (25). IL-18 promotes articular Th1 responses and is thus important in the pathophysiology of RA (21).

Integral to the inflammation in RA is the process of angiogenesis. We found that IL-18 induced endothelial migration in the HMVEC migration assay, which is one aspect of angiogenesis in vitro. IL-18 induced HMVEC migration in the nanomolar range in......
TNF-α up-regulates the production of other proangiogenic cytokines, such as IL-8, which was more potent than or comparable with IL-18. The HMVEC migration assay revealed that IL-8 was a more potent IL-8 and ENA-78 in the HMVEC migration of RA SFs. These results indicate the angiogenic role of IL-18 in endothelial cell migration in RA.

To compare the contribution of RA SF IL-18 with other mediators of RA SF that also induce endothelial cell migration, we further immunodepleted RA SFs of IL-18 alone and in combination with the chemokines IL-8 or ENA-78 (12, 43). IL-18 appeared to account for a significant portion of chemotactic activity for HMVECs in the SFs comparable with that by IL-8. The contribution by IL-18 was 1.6-fold greater than that by ENA-78. Combining immunodepletions of IL-18 and IL-8 or ENA-78 did not augment suppression of HMVEC migration. The lack of further suppression suggests that inhibiting the effect of one of these cytokines may be blocking common pathways involved in HMVEC migration. IL-18 accounted for approximately two-thirds of the migrating activity of HMVECs in RA SFs comparable with that by IL-8. The contribution of RA SF IL-18 with other mediators of RA SF that also induce endothelial cell migration, we further immunodepleted RA SFs of IL-18 alone and in combination with the chemokines IL-8 or ENA-78 (12, 43). IL-18 appeared to account for a significant portion of chemotactic activity for HMVECs in the SFs comparable with that by IL-8. The contribution by IL-18 was 1.6-fold greater than that by ENA-78. Combining immunodepletions of IL-18 and IL-8 or ENA-78 did not augment suppression of HMVEC migration. The lack of further suppression suggests that inhibiting the effect of one of these cytokines may be blocking common pathways involved in HMVEC migration. IL-18 accounted for approximately two-thirds of the migrating activity of HMVECs in RA SFs comparable with that by IL-8. This finding supports a potent role of IL-18 in endothelial cell migration in RA.

To elucidate a mechanism by which IL-18 mediates angiogenesis, we examined the potential role of the integrin αβ1 in HMVEC migration. Endothelial cell invasion, migration, and proliferation are regulated in part by the integrin family of cell adhesion molecules, and we have shown that αβ1 is up-regulated on RA compared with osteoarthritic or normal synovial blood vessels (48). We therefore hypothesized that bFGF and TNF-α (34), IL-18 may also act via this integrin in angiogenesis. After blocking αβ1 on HMVECs with specific anti-αβ1 Ab, the degree of IL-18-induced cell migration was inhibited by 34% compared with blocking with isotype-matched control Ab. αβ1 antagonists have successfully modulated angiogenesis in a rabbit model of synovitis (49). Targeting IL-18, which appears to function in part through this integrin, could be an alternative therapeutic approach for RA.

Endothelial cell proliferation is another aspect of angiogenesis. We did not demonstrate direct inhibition or enhancement of basal endothelial cell (HMVEC) proliferation, which is in contrast to previous reports of the antimitogenic activity of IL-18. IL-18 at 1–10 nM concentration has been reported to inhibit FGF-2-induced capillary endothelial cell proliferation (50). However, the effect of IL-18 on basal proliferation, as examined in our study, was not explored. This group also found that murine, not human, IL-18 suppressed FGF-2-induced corneal neovascularization in mice, although the IL-18 was administered by i.p. injections, and it is unclear whether local rather than systemic treatment would have induced divergent results. Interestingly, they also found that IL-18 suppressed angiogenesis in the chick chorioallantoic membrane model. Our finding that IL-18 did not induce HMVEC proliferation is consistent with results obtained for other angiogenic mediators, such as soluble VCAM-1 or soluble E-selectin, which also do not induce endothelial proliferation (14).

Consistent with the chemotactic properties of IL-18, we also showed IL-18 induction of HMVEC tube formation in Matrigel matrix in vitro. The degree of tube formation by IL-18 at 1 nM was comparable with the stimulant PMA (50 nM), a potent inducer of HMVEC differentiation and tube formation (51). In the Matrigel in vivo mouse angiogenesis model, IL-18 also stimulated actual blood vessel formation, which was visible in excess of control. The potency of IL-18 relative to other angiogenic stimulants in this assay can be assessed, since 1 nM (or 18 ng/ml) IL-18 produced results similar to that induced by 1 ng/ml aFGF, which is clearly one of the strongest angiogenic stimuli. By comparison, an excess of 100-fold more bFGF would be required to produce the same effect in this assay as 1 ng/ml aFGF (0.86 g/dl vs 1.30 g/dl hemoglobin), and 10-fold more TNF-α (10 ng/ml hemoglobin) would be expected to produce twice the effect as 1 ng/ml aFGF (2.3 g/dl vs 1.3 g/dl hemoglobin) (37).

The Matrigel in vivo assay also allowed for another examination of a potential mechanism behind the angiogenic activity of IL-18. The angiogenic effect of IL-18 in the presence of anti-TNF-α Ab was evaluated by adding IL-18 and neutralizing anti-TNF-α Ab to the Matrigel before injection. Although the endothelial cells were the target of the effect of IL-18 in cell migration and tube formation observed in the earlier assays, the new blood vessel formation in the Matrigel plug could involve cellular targets other than endothelial cells, at least indirectly. In our hands, blocking the local TNF-α did not significantly alter the effect of IL-18 on Matrigel plug hemoglobin content. However, it is not clear whether systemic inhibition of TNF-α would have diminished the effect of IL-18. In addition, the downstream effect of IL-18 on PBMCs, lymphocytes, and other cells via up-regulation of other cytokines cannot be overlooked. The various mediators known to orchestrate the angiogenic process may function in part through common pathways. Although redundancy in the system exists and serves to
maintain the angiogenic state characteristic of certain physiologic or pathologic conditions, evidence from clinical trials clearly demonstrates that blocking one key element through treatment targeting TNF-α alone reduced RA disease activity (52). Further studies examining potential mechanisms of angiogenesis, including common signaling pathways, cell adhesion, andup-regulation of cytokines, integrins, and other mediators, may shed light on the significance of the current findings.

The sponge angiogenesis system allows testing of the effect of various antagonists and agonists on the proliferation of blood vessels and stroma, provides histological and quantitative information, and is easily reproducible. This system applied to mice was suitable to test the independent effect of IL-18 on angiogenesis, and specifically, new blood vessel formation, which this assay measures by virtue of the constant sample area of the disc and the planar growth of the vessels (41). IL-18 at 10 nM strongly induced angiogenesis, as measured by a 5-fold increase in the amount of hemoglobin over control, similar to the 4-fold increase seen with the positive control stimulant aFGF (1 nM). This finding not only confirms our results from the Matrigel plug in vivo assay, but it establishes IL-18 as a direct angiogenic stimulus.

In summary, we found that IL-18 induces endothelial cell chemotaxis, and that IL-18 accounts for a significant portion of the hemoglobin over control, similar to the 4-fold increase seen with the positive control stimulant aFGF (1 nM). This finding not only confirms our results from the Matrigel plug in vivo assay, but it establishes IL-18 as a direct angiogenic stimulus.

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References


