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In Vivo Inhibition of Angiogenesis by Interleukin-13 Gene Therapy in a Rat Model of Rheumatoid Arthritis

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Objective. **Interleukin-13 (IL-13) is a pleiotropic cytokine that can affect vessel formation, an important component of the rheumatoid arthritis (RA) synovial tissue pannus. The purpose of this study was to use a gene therapy approach to investigate the role of IL-13 in angiogenesis in vivo, using a rat adjuvant-induced arthritis model of RA.**

Methods. **Ankle joints of female rats were injected preventatively with an adenovirus vector containing human IL-13 (AxCAIL-13), a control vector with no insert (AxCANI), or phosphate buffered saline (PBS). Joints were harvested at the peak of arthritis, and histologic and biochemical features were evaluated.**

Results. **AxCAIL-13–treated joint homogenates had lower hemoglobin levels, suggesting reduced joint vascularity, and both endothelial cell migration and tube formation were significantly inhibited ($P < 0.05$). Similarly, AxCAIL-13 inhibited capillary sprouting in the rat aortic ring assay and vessel growth in the Matrigel plug in vivo assay. IL-13 gene delivery resulted in up-regulation and association of phosphorylated ERK-1/2 and protein kinase Cα/βII, suggesting a novel pathway in IL-13–mediated angiostasis. The angiostatic effect of AxCAIL-13 was associated with down-regulation of proangiogenic cytokines (IL-18, cytokine-induced neutrophil chemoattractant 1/CXCL1, lipopolysaccharide-induced CXC chemokine/CXCL5) and up-regulation of the angiogenesis inhibitor endostatin. The expression and activity of matrix metalloproteinases 2 and 9, which participate in angiogenesis, was impaired in response to IL-13 as compared with AxCANI and PBS treatment.**

Conclusion. **Our findings support a role for IL-13 as an in vivo antiangiogenic factor and provide a rationale for its use in RA to control pathologic neovascularization.**

Angiogenesis is a highly orchestrated process involving the sprouting of new capillary-like structures from existing vasculature that mature into a system of new blood vessels, resulting in neovascularization (1). It can be triggered and modified by a number of factors, including cytokines, chemokines, growth factors, and matrix metalloproteinases (MMPs) (2). Angiogenesis is central to many pathophysiologic conditions, such as tumor growth, diabetic retinopathy, and rheumatoid arthritis (RA) (1,3). The angiogenic process is often of pivotal importance in the progression of disease and is thus an interesting and promising therapeutic target (4). Indeed, treatment of tumors and of RA in animal models with vectors expressing interleukin-4 (IL-4), IL-13, IL-10, and IL-1 antagonists using a gene therapy

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approach have shown success (5,6). Therefore, we investigated the possibility that IL-13 modulates arthritis directly by inhibiting the angiogenic process.

IL-13 is a multifunctional cytokine with some antiinflammatory effects in the context of inflammatory arthritis (7). We have previously shown in rat adjuvant-induced arthritis (AIA) that either human IL-13 (AxCAIL-13) or rat IL-13 (AxCArIL-13) administered via an adenovirus vector profoundly reduced joint swelling and local chemokine production (7). However, its specific role and mechanisms in neovascularization remain unclear. What is known is that IL-13 signals through type I cytokine receptors that form heterodimers and have binding domains for JAKs (8). Accordingly, the biologic effects of IL-13 are often mediated via the JAK/STAT pathway or via insulin receptor substrate 1 (IRS-1) and IRS-2 on the IL-4 receptor subunit (9). However, recent studies suggest that MAPK pathways may play a critical role in effector responses of IL-13, including inflammation and tissue remodeling (10,11), but their role in IL-13–mediated effects on angiogenesis remains to be determined.

We have shown that IL-13 has both stimulatory and inhibitory effects on endothelial cell (EC) migration in vitro, a critical step in angiogenesis, pointing to a dose-dependent phenomenon (12,13). Fukushi et al (14) demonstrated that IL-13 significantly stimulated the formation of tube-like structures by ECs in vitro and led to corneal neovascularization in rats. In contrast, we have previously shown that IL-13 not only reduced inflammation, but also attenuated vascularization of the synovium in AIA in the rat, an established model of RA (7). Thus, to clarify the participation of IL-13 and to elucidate its mechanism of action in joint inflammation, we used a gene therapy approach to investigate the role of IL-13 on angiogenesis in vivo, using rat AIA as a model for neovascularization.

IL-13 inhibits the production of monocyte and other cell-derived proinflammatory mediators, such as cytokines, that in turn, can affect the angiogenic response (9,15). Recently, we demonstrated that reduced synovial vascularization in response to IL-13 gene delivery resulted in impaired joint levels of monocyte chemotactic protein 1 (MCP-1) (7), a potent proangiogenic chemokine (16,17). Among the various processes that regulate angiogenesis, pericellular proteolysis is a precondition for EC migration and capillary tube formation in vivo (18,19), a process mainly regulated by MMPs. Among the MMPs, the gelatinases MMP-2 and MMP-9 and the membrane-type MMPs have been suggested to play a key role in angiogenesis (20,21).

In this study, we demonstrate that IL-13 gene therapy inhibits angiogenesis in vivo by way of a novel signaling pathway via protein kinase Cα/βII (PKCα/βII) and ERK-1/2. The angiostatic effect of IL-13 is associated with a shift in the balance of pro- and antiangiogenic cytokines and decreased expression and activity of MMP-2 and MMP-9.

MATERIALS AND METHODS

Preparation of adenovirus and experimental setup. Replication-incompetent adenoviruses producing human IL-13 (AxCAIL-13) or having no foreign gene insert (AxCANI) were prepared, and AIA was induced in female Lewis rats as described previously (7). AxCAIL-13 or rat AxCArIL-13 was shown to successfully suppress arthritis clinically (7). Rats were divided into 3 groups: one group received AxCAIL-13, the second received AxCANI (control), and the third received phosphate buffered saline (PBS; control). The AxCANI group was included to distinguish between the effects of the adenovirus and the effects of the IL-13. On day 8 after AIA induction, prior to development of arthritis, PBS or 5×10^6 plaque-forming units of either AxCAIL-13 or AxCANI was administered to each hind ankle via intraarticular injection. All animals were euthanized on day 18 after adjuvant injection, at the peak of inflammation, and the ankles were collected for further analysis.

Preparation of ankle homogenates and determination of hemoglobin and protein concentrations. For angiogenesis assays, rat joints were homogenized as described previously (22). Hemoglobin levels were measured by the tetramethylbenzidine method. A bicinchoninic acid assay (BCA Protein Assay kit; Pierce, Rockford, IL) was used to determine protein concentrations.

Cell culture. Human dermal microvascular endothelial cells (HMVECs; Cambrex, Walkersville, MD) were maintained in EC basal medium (EBM) supplemented with 10% fetal bovine serum (FBS) and additional growth factors (Cambrex) at 37°C in an atmosphere of 5% CO_2. Cells between passages 5 and 12 were used for angiogenesis assays. For some studies, human U937 cells were cultured for signal transduction studies. Briefly, U937 cells were cultured in suspension to $\sim 10^6$ cells/ml in T175 flasks with 10% FBS in RPMI 1640 containing penicillin and streptomycin.

In vitro migration of HMVECs. HMVEC in vitro migration was determined using a modified 48-well Boyden chamber as previously described (2). Pooled joint homogenates from 5 rats (60 μg of protein/ml), PBS, or positive control basic fibroblast growth factor (bFGF; 60 n*M*) were used as stimulant. Each assay was run in quadruplicate, and the results were expressed as the number of HMVECs migrating per high-power field (hpf; 40× magnification).

For some studies, neutralizing antibody to human IL-13 was incubated with pooled ankle homogenates obtained on day 18 from AxCAIL-13–treated rats to evaluate inhibition of HMVEC migration due solely to human IL-13. AxCAIL-13–treated rat joint homogenates (60 μg/ml) were used in all samples, and homogenates were combined with either normal

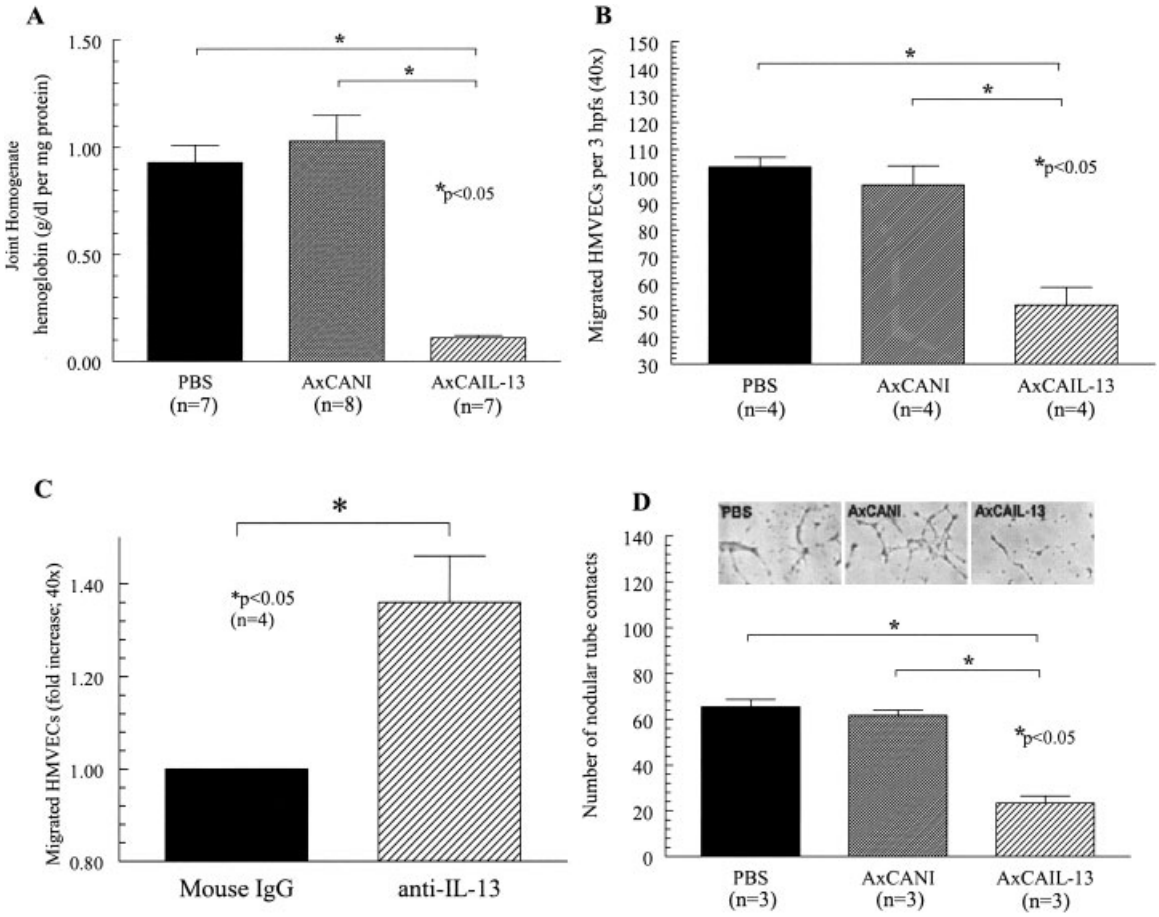

Figure 1. Inhibition of angiogenesis in joint homogenates from rats with adjuvant-induced arthritis (AIA) treated with AxCAIL-13. **A,** Hemoglobin levels were measured in ankle homogenates and normalized to the total joint protein content. Hemoglobin levels were significantly lower following AxCAIL-13 treatment as compared with phosphate buffered saline (PBS) and AxCANI, which emphasizes the angiostatic effect of interleukin-13 (IL-13) in vivo. Values are the mean and SEM of (*n* = number of joint homogenates). **B,** AxCAIL-13 joint homogenates significantly inhibited human dermal microvascular endothelial cell (HMVEC) migration compared with PBS and AxCANI homogenates. Values are the mean and SEM (n = number of assays). HMVEC migration in response to PBS alone (mean ± SEM 54.4 ± 7.2 cells/high-power field [hpf]) or to 60 n*M* basic fibroblast growth factor (112.8 ± 14.1 cells/hpf) served as negative and positive controls, respectively (data not shown). **C,** Neutralizing antibody against IL-13 reversed the inhibition of HMVEC migration by ankle homogenates obtained on day 18 from AxCAIL-13–treated rats. AxCAIL-13 joint homogenates (60 mg/ml) were the chemoattractant in all samples. Homogenates were combined with 10 μg/ml of either normal mouse IgG or neutralizing mouse anti-human IL-13 before addition to Boyden chambers containing HMVECs. Values are the mean and SEM (n = number of assays). **D,** Rat AIA joint homogenate–induced EC tube formation in an in vitro Matrigel assay was inhibited by IL-13 gene therapy. Values are the mean and SEM (n = number of plugs per group). **Inset,** Representative photomicrographs showing EC tube formation in the 3 treatment groups (original magnification × 40).

mouse IgG or neutralizing mouse anti-human IL-13 antibody (10 μg/ml respectively; R&D Systems, Minneapolis, MN).

HMVECs and homogenates were added to the Boyden chambers, and the numbers of migrating cells were counted after a 2-hour incubation period.

In vitro capillary morphogenesis assay. Tube formation by HMVECs in growth factor–reduced (GFR) Matrigel was used to evaluate the effect of AxCAIL-13–treated rat joint homogenates on capillary morphogenesis, as previously described (23). Eight ankle homogenates per group (60 μg of protein/ml) were tested as stimulant. Phorbol myristate acetate (PMA; 50 n*M*) and DMSO served as controls. After 16–18 hours, capillary morphogenesis was examined under a phase-contrast microscope, and node formation (defined as a nodular contact formation of at least 3 adherent endothelial cell tubes) was evaluated blindly. The assay was performed 3 times.

Growth assay for rat aortic ring microvessel sprouting. Aortas from female Sprague-Dawley rats (100 gm) were

dissected and sliced into 1-mm–thick rings (24). Aortic rings were placed into 300 μl of GFR Matrigel in 48-well plates. Serum-free EBM medium (300 μl) with pooled joint homogenates (60 μg of protein/ml) from rats with AIA was added and replaced 3 times each week. PMA (50 n*M*) and DMSO were used as positive and negative controls, respectively. Microvessel sprouting from the adventitial layer was assessed and scored using the following scale (25): 0 = no sprouting, 0.25 = isolated sprouting, 0.5 = sprouting in 20–50% of the arterial circumference, 1 = sprouting in 50–75% of the circumference, 1.5 = sprouting in 100% of the circumference, and 2 = 100% of the arterial circumference occupied by sprouts longer than one-third of the length of the average radius of the rings.

Matrigel plug in vivo angiogenesis assay. To examine the effects of AxCAIL-13 treatment on angiogenesis in vivo, Matrigel plug assays were performed as previously described (26). GFR Matrigel containing either pooled joint homogenates (120 μg of protein/ml) from rats with AIA, PBS, or bFGF (1 ng/ml) was injected subcutaneously into female C57BL/6 mice. After 7 days, the plugs were dissected, homogenized, and analyzed. Hemoglobin and protein levels were measured as described above. In some experiments, Matrigel (500 μl) containing homogenates from rat ankles treated with AxCAIL-13 or AxCANI (120 μg/ml of protein) was injected subcutaneously into the C57BL/6 mice. After 7 days, the Matrigel plugs were removed, embedded in OCT, cryosectioned, and stained with Diff-Quick, and the number of blood vessels was counted by an observer (MAA) who was blinded to the experimental setup.

Enzyme-linked immunosorbent assays (ELISAs) for rat cytokines and growth factors. Commercially available ELISA kits detecting the following proteins were used to determine possible mediators or inhibitors of angiogenesis in joint homogenates from rats with AIA: fractalkine/CX_3CL1, cytokine-induced neutrophil chemoattractant 1 (CINC-1)/CXCL1, lipopolysaccharide-induced CXC chemokine (LIX)/CXCL5, IL-6, IL-18, endostatin, and the total (latent plus active) amount of transforming growth factor β (TGFβ). The sensitivity of these ELISAs is routinely 15 pg/ml. Levels of CXCL16 were analyzed by a sandwich ELISA (27), with a sensitivity of 125 pg/ml.

Western blot analysis and immunoprecipitation. Equal protein amounts of rat AIA joint homogenates were subjected to 10% sodium dodecyl sulfate (SDS) gel electrophoresis, transferred onto nitrocellulose membranes, and subjected to Western blot analysis as previously described (28), using the following rabbit anti-human antibodies to selected phosphorylated (*p) signaling molecules (all from Cell Signaling Technology, Beverly, MA): anti–*p–ERK-1/2, anti-*pJAK1, anti–*p-JNK, anti–*p–NF-κB p65, anti–*p–p38 MAPK, anti-*pp70S6K, anti–*p-PKCα/βII, anti–*p-Raf, anti–*p-Src, anti-*pSTAT1, and anti-*pSTAT6. Subsequently, blots were reprobed with rabbit antiactin (Sigma, St. Louis, MO) or anti–total NF-κB (Santa Cruz Biotechnology, Santa Cruz, CA) antibody. Bands were scanned, and their intensities were analyzed with Un-Scan-It software version 5.1 (Silk Scientific, Orem, UT). Immunoprecipitation was performed as described previously (29), using antiphosphotyrosine or anti–ERK-1/2 (Cell Signaling Technology), followed by immunoblotting.

To further understand and authenticate the mechanism through which IL-13 modulation of the signaling molecules inhibits inflammation and angiogenesis in the joints of the treated rats, we used the U937 monocytic cell line to study the effects of IL-13 on the phosphorylation of the proteins of interest. Monocyte/macrophages are producers of many potent angiogenic mediators in the human RA joint (30,31). U937 cells (1×10^6/ml) in 6-well plates in 5% FBS/RPMI 1640 were treated or were not treated with recombinant human IL-13 at a concentration range of 2.5–20 ng/ml for 20 minutes. Cells were lysed in lysis buffer (100 m*M* Tris, pH 7.4, 100 m*M* NaCl, 1 m*M* EDTA, 1 m*M* EGTA, 1 m*M* NaF, 20 m*M* NaH_2PO_4, 2 m*M* Na_3VO_4, 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5% deoxycholate, 1 m*M* phenylmethylsulfonyl fluoride) and protease inhibitors (1 tablet/10 ml; Roche Diagnostics, Indianapolis, IN), and protein was measured using BCA Protein Assay kits (Pierce, Rockford, IL). Equal amounts of protein (20 μg) were separated by SDS–polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (Bio-Rad). Western blot analysis was performed as previously described (28).

Gelatin zymography and immunofluorescence. MMP activity in homogenates from rats with AIA was identified by gelatin zymography. MMP-2 and MMP-9 activity presented as digested unstained bands on the gelatin gels, according to the molecular weights of proMMP-9 (92 kd), proMMP-2 (72 kd), and active MMP-9 (62 kd). Photographs were taken with a Nikon Coolpix 4500 digital camera (Nikon, Melville, NY). For immunofluorescence studies, HMVECs were plated overnight at 20,000 cells/well in 8-well chamber slides in EBM with 5% FBS. Cells were serum-starved for 1 hour and stimulated with joint homogenates from rats with AIA (30 μg protein/ml) for 0, 5, and 30 minutes and analyzed using goat anti–MMP-2 or anti–MMP-9 antibodies (Santa Cruz Biotechnology) and Alexa Fluor 488–conjugated secondary antibody (Invitrogen, Carlsbad, CA). Images were taken using an Olympus BX51 Fluorescence Microscope System with DP Manager imaging software (Olympus, Lake Success, NY).

Statistical analysis. Data are reported as the mean ± SEM. For statistical purposes, each ankle was treated independently, since AIA in rats often develops independently in each ankle (22). Statistical analysis was performed using Student's *t*-test. P values less than 0.05 were considered statistically significant.

RESULTS

Reduced numbers of functional vessels in vivo following IL-13 gene delivery in rats with AIA. To determine if IL-13 gene delivery reduces vascularity in the joints of rats with AIA, hemoglobin levels, which are proportional to the number of functional vessels in the tissue, were measured in ankle homogenates (Figure 1A). Hemoglobin levels were significantly lower in the AxCAIL-13 group (mean ± SEM 0.11 ± 0.01 gm/dl per mg of protein) than in the PBS and AxCANI groups (0.93 ± 0.08 and 1.03 ± 0.12, respectively; $P < 0.05$ for each comparison).

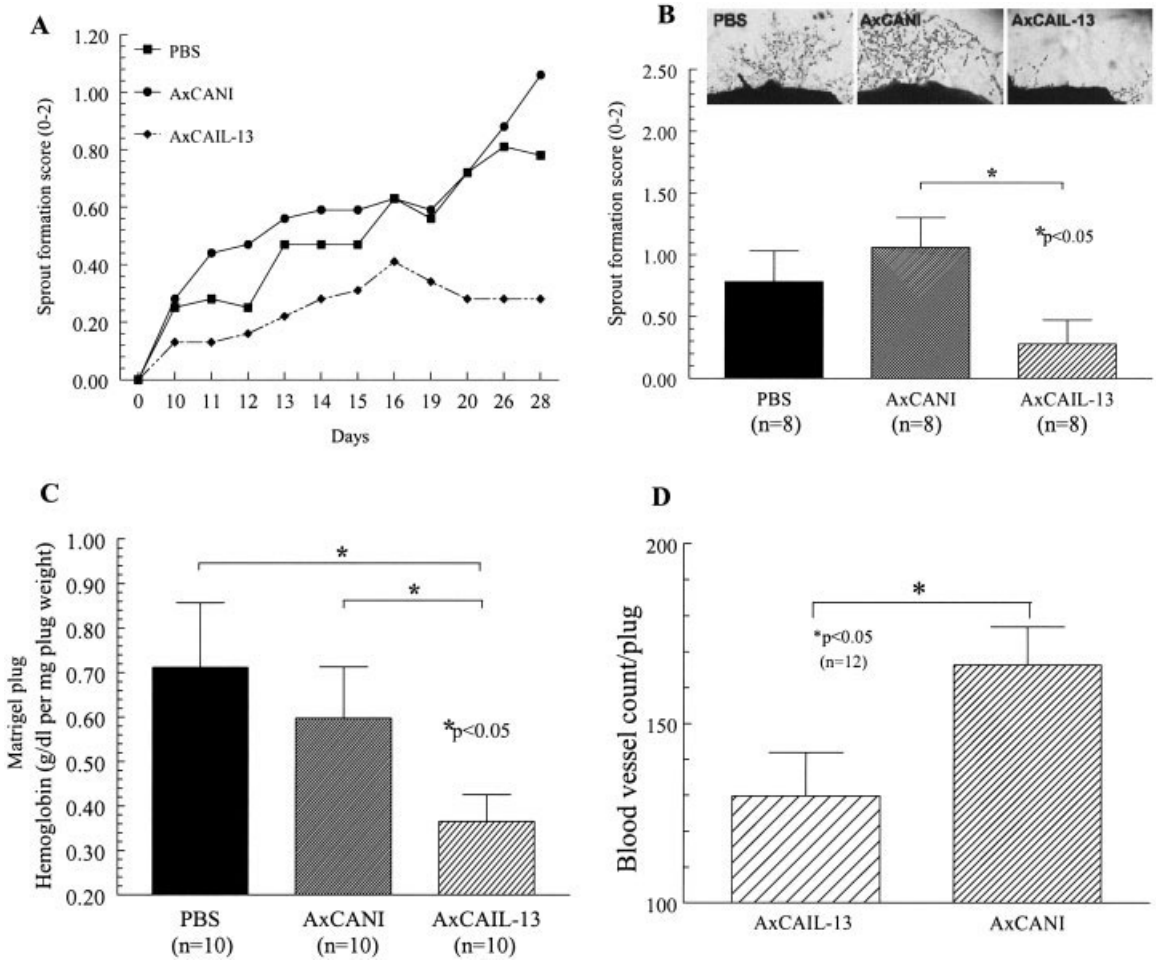

Figure 2. Inhibition of angiogenesis ex vivo and in vivo by AxCAIL-13. To assess the effect of AxCAIL-13 gene therapy on complex angiogenic processes, a rat aortic ring assay and a Matrigel angiogenesis in vivo plug assay were performed. **A,** Vessel sprouting induced by joint homogenates from rats with adjuvant-induced arthritis (AIA), starting after ~10 days, was inhibited by AxCAIL-13, but not AxCANI or phosphate buffered saline (PBS). Values are the mean of 8 aortic rings per group. **B,** Sprout formation on day 28 was significantly less responsive to AxCAIL-13–treated rat AIA joint homogenates than to AxCANI–treated joint homogenates. Values are the mean and SEM (n = number of joint homogenates). **Inset,** Representative photomicrographs showing vessel sprouting in the 3 treatment groups (original magnification × 40). Phorbol myristate acetate–induced sprouting (mean ± SEM score 0.94 ± 0.39; n = 4) was similar to that in the PBS and AxCANI group, and was significantly higher than that with DMSO (score of 0.06 ± 0.06; n = 4 [$P < 0.05$]) (data not shown). **C,** In the Matrigel angiogenesis in vivo plug assay, hemoglobin levels in response to AxCAIL-13 (0.37 ± 0.06 gm/dl per mg of plug weight) were significantly lower than those in response to PBS and AxCANI, but were similar to those with Matrigel plugs and only PBS (negative control; score of 0.38 ± 0.21) (data not shown). Values are the mean and SEM (n = number of mice). **D,** To further address the antiangiogenic properties of interleukin-13 (IL-13) gene therapy in vivo, Matrigel (500 μl) containing homogenates obtained on day 18 from rat ankles treated with human AxCAIL-13 or AxCANI (120 μg/ml of protein) was subcutaneously injected into C57BL/6 mice. After 7 days, Matrigel plugs were removed, embedded in OCT, cryosectioned, stained with Diff-Quick, and blood vessels were counted. Plugs containing AxCAIL-13 homogenates showed significantly less blood vessel development than did those containing AxCANI homogenates. Values are the mean and SEM (n = number of mice).

Inhibition of in vitro EC migration following IL-13 gene delivery. We first tested in vitro whether joint homogenates from rats with AIA induce EC migration, a crucial step in the process of neovascularization, and whether IL-13 gene therapy is able to impair this effect (Figure 1B). HMVEC migration in response to homogenates from PBS-treated AIA rats was ~2-fold higher than baseline and was in the range of migration in

response to bFGF (data not shown). HMVEC chemotaxis in vitro in response to AxCANI (mean ± SEM 96.8 ± 7.1 cells/3 hpf) was comparable to the effect induced by the PBS homogenates (103.5 ± 3.6), whereas AxCAIL-13 significantly inhibited the migratory response compared with both groups (52.0 ± 6.6; $P < 0.05$), suggesting that IL-13 gene delivery inhibits EC migration.

We further tested the effects of neutralizing antibody against human IL-13 to see if this would reverse the inhibition of HMVEC migration by day 18 in AxCAIL-13–treated rat ankle homogenates. This would show specificity of human IL-13 to directly inhibit HMVEC chemotaxis in vitro. As shown (Figure 1C), AxCAIL-13–treated rat joint homogenates incubated with nonspecific mouse IgG reduced HMVEC chemotaxis, as measured in a modified Boyden chemotaxis system. However, homogenates combined with neutralizing mouse anti-human IL-13 abrogated the inhibitory effect, demonstrating that inhibition of angiogenesis by IL-13 can be reversed by neutralization of human IL-13 in vitro. These results indicate that inhibition of HMVEC chemotaxis can be directly attributed to IL-13 expression. Negative and positive controls were PBS (8.9 ± 0.7 cells/3 hpf) and bFGF (16.8 ± 1.2 cells/3 hpf), respectively. Results are expressed as the fold increase compared with control IgG.

AxCAIL-13–induced reduction of EC tube formation in an in vitro capillary morphogenesis assay. To examine the role of in vivo IL-13 gene therapy on EC differentiation, another important step in angiogenesis, we studied EC tube formation in an in vitro capillary morphogenesis assay in response to PBS-, AxCANI-, and AxCAIL-13–treated joint homogenates from rats with AIA (Figure 1D). Joint homogenate–induced tube formation (mean ± SEM 65.5 ± 3.2 nodular tube contacts) was not altered by AxCANI (61.8 ± 2.2), but AxCAIL-13 treatment resulted in a joint environment that significantly impaired EC tube formation (23.4 ± 3.1; $P < 0.05$). These data imply that IL-13 gene delivery opposes EC differentiation.

AxCAIL-13–induced inhibition of vessel sprouting stimulated by joint homogenates from rats with AIA, by aortic ring assay. To confirm the in vitro data and to test the effect of AxCAIL-13 on the concerted action of both EC migration and differentiation, we next performed a rat aortic ring assay to determine whether joint homogenates from rats with AIA treated with AxCAIL-13 are less likely than control AxCANI-treated or PBS-treated homogenates to promote EC sprouting (Figures 2A and B). EC sprout formation started after ~10 days in the PBS group, constantly increasing until day 28, thereby confirming that rat AIA joint homogenates have proangiogenic properties in this assay. While treatment with AxCANI did not alter the extent of sprout formation, no significant sprouting was observed with AxCAIL-13 joint homogenates (Figure 2A). Indeed, on day 28, sprout formation scores in the AxCAIL-13 group were significantly reduced in comparison with the AxCANI group (mean ± SEM score 0.28 ± 0.19 versus 1.06 ± 0.24; $P < 0.05$) and tended to be higher than in the PBS group (Figure 2B). PMA-induced sprouting (score of 0.94 ± 0.39) was comparable to that in the PBS and AxCANI group and was significantly higher than that in the DMSO group used as a negative control (score of 0.06 ± 0.06; $P < 0.05$) (data not shown).

AxCAIL-13–induced inhibition of angiogenesis in vivo. To further evaluate the antiangiogenic effects of IL-13 gene therapy in an in vivo setting, we performed a Matrigel plug in vivo angiogenesis assay, in which hemoglobin levels are determined in order to assess the extent of blood vessel penetration and formation in response to stimulus (Figure 2C). The hemoglobin content of the PBS-treated ankle homogenates (mean ± SEM 0.71 ± 0.15 gm/dl per mg of plug weight) was increased ~1.9-fold as compared with Matrigel plugs containing only PBS (data not shown), but was similar to that in the AxCANI group (0.60 ± 0.12 gm/dl per mg of plug weight). AxCAIL-13 treatment resulted in significantly decreased hemoglobin levels (0.37 ± 0.36 gm/dl per mg of plug weight; $P < 0.05$), suggesting an important role in the inhibition of blood vessel growth in arthritis in vivo.

To examine actual vessel growth in vivo, Matrigel (500 μl) containing day 18 homogenates from rat ankles treated with either human AxCAIL-13 or AxCANI (120 μg/ml of protein) was subcutaneously injected into C57BL/6 mice. Matrigel plugs containing AxCAIL-13 homogenates showed significantly less blood vessel development as compared with the plugs containing AxCANI homogenates ($P < 0.05$) (Figure 2D).

Activation of PKCα/βII and ERK-1/2 following IL-13 gene therapy. To identify possible signaling pathways involved in IL-13–mediated antiangiogenic effects in vivo, we analyzed the expression of different phosphorylated signaling molecules in rat AIA joint homogenates, using immunoprecipitation and immunoblotting techniques (29) (Figure 3 and Table 1). We studied the expression of *p-Tyk2 and *pSTAT6, signaling molecules known to be activated by IL-13, by immunoprecipitation and/or immunoblotting techniques. Both *p-

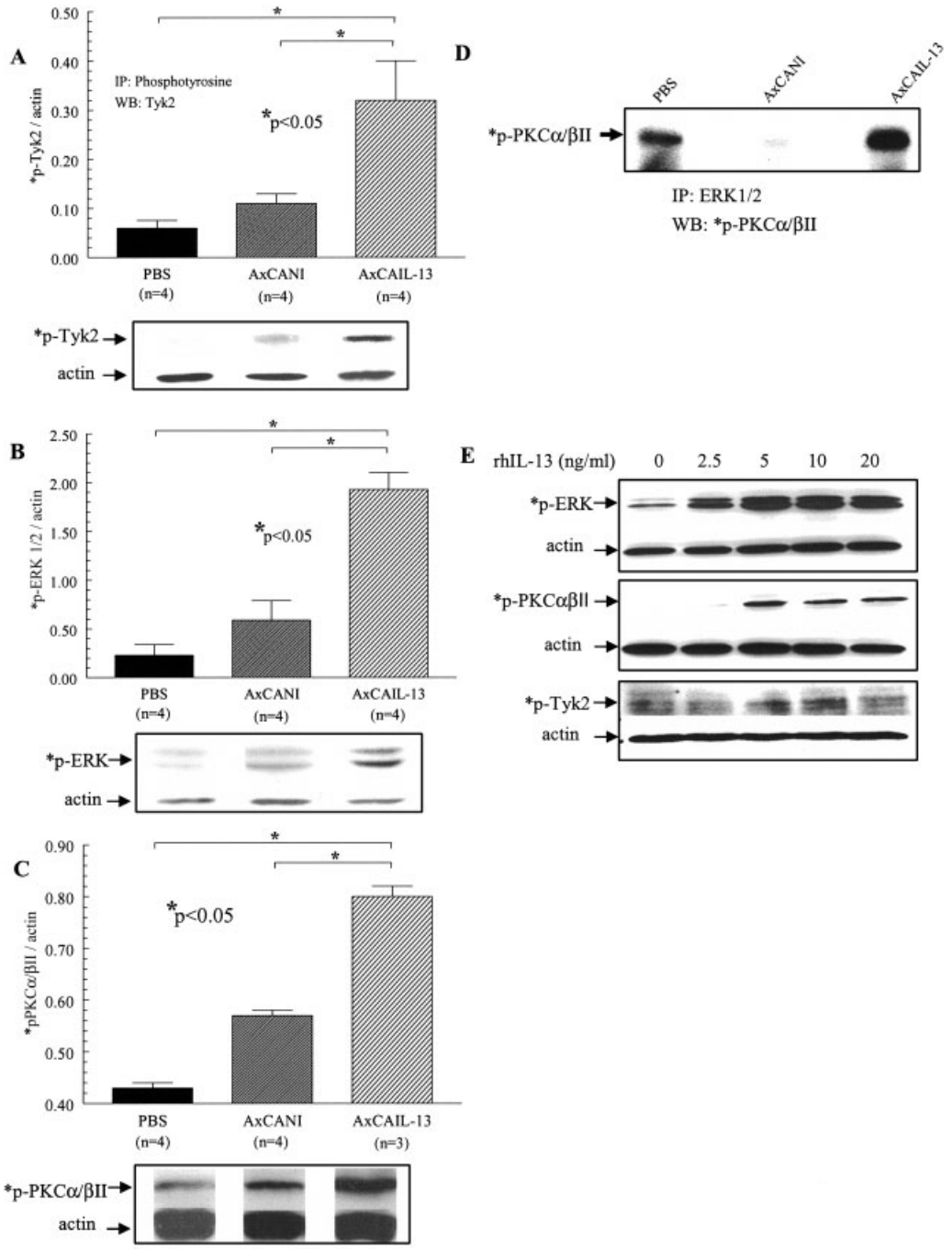

Figure 3. Induction of ERK-1/2 and protein kinase C (PKC) phosphorylation by interleukin-13 (IL-13) gene therapy. **A,** Immunoprecipitation (IP) of total ankle homogenates with phosphotyrosine and subsequent Western blotting (WB) for Tyk2 showed a significant up-regulation of *p-Tyk2 in response to IL-13 gene therapy in vivo. **B,** AxCAIL-13 increased the amount of *p–ERK-1/2 as compared with phosphate buffered saline (PBS) and AxCANI, as determined by immunoblotting. **C,** AxCAIL-13 treatment resulted in up-regulation of *p-PKCα/βII, as determined by Western blotting. Values in **A–C** are the mean and SEM (n = number of joint homogenates). **D,** Immunoprecipitation with ERK-1/2 and immunoblotting with *p-PKCα/βII showed up-regulation in the AxCAIL-13 group, suggesting an association between the 2 signaling molecules. Joint homogenates from rats with adjuvant-induced arthritis were used for the studies shown in **A–D**. **E,** Recombinant human IL-13 (rhIL-13) dose-dependently induced the phosphorylation of Tyk2, ERK-1/2, and PKCα/βII in U937 monocytic cells. Western blots shown are representative of 3 independent experiments.

Table 1. Densitometry of Western blots detecting signaling molecule expression in ankle joint homogenates from rats with adjuvant-induced arthritis preventatively injected with AxCANI or AxCAIL-13*

	Rats injected with AxCANI	Rats injected with AxCAIL-13
Up-regulated signaling molecules		
*p–ERK-1/2	0.59 ± 0.02 (4)	1.93 ± 0.17 (4)†
*p-PKCα/βII	0.57 ± 0.01 (4)	0.80 ± 0.02 (3)†
*p-Raf	0.024 ± 0.003 (4)	0.073 ± 0.015 (3)†
*pSTAT6	0.44 ± 0.01 (5)	0.97 ± 0.02 (5)†
*p-Tyk2	0.11 ± 0.02 (4)	0.32 ± 0.08 (4)†
Unchanged signaling molecules		
*p-Akt	0.09 ± 0.03 (4)	0.19 ± 0.05 (4)
*p-Src	0.16 ± 0.01 (4)	0.28 ± 0.06 (3)
*pJAK1	0.03 ± 0.004 (4)	0.025 ± 0.007 (3)
*p-JNK	0.30 ± 0.03 (4)	0.20 ± 0.04 (3)
*p–p38 MAPK	0.07 ± 0.02 (4)	0.04 ± 0.01 (4)
*pp70S6K	0.04 ± 0.003 (3)	0.03 ± 0.005 (4)
*pSTAT1	0.37 ± 0.04 (5)	0.45 ± 0.03 (5)
Down-regulated signaling molecules		
*p–NF-κB p65	0.38 ± 0.05 (3)	0.10 ± 0.02 (4)

* Joint homogenates obtained on day 18 were used for all Western blot analyses. Values are the mean ± SEM ratio of signaling molecule to actin; values in parentheses are the number of ankles. PKCα/βII = protein kinase Cα/βII.
† $P < 0.05$ versus AxCANI-injected group.

Tyk2 and *pSTAT6 were significantly increased in response to IL-13 gene therapy as compared with AxCANI or PBS treatment (Figure 3A and Table 1). Of note, we found a strong up-regulation of *p–ERK-1/2 and *p-PKCα/βII in the AxCAIL-13 group (Figures 3B and C). In addition, up-regulation of *p-Raf (Table 1) emphasized the notion of an IL-13–mediated activation of the ERK-1/2 pathway. The expression of *p-Akt, *p-Src, *pJAK1, *p-JNK, *p–p38 MAPK, *pp70S6K, and *pSTAT1 were unchanged. Interestingly, the expression of *p–NF-κB p65 was significantly down-regulated, indicating reduced transcription activity via NF-κB, and *pSTAT1 expression was not modified in response to AxCAIL-13 (Table 1).

To test whether the phosphorylation steps of ERK-1/2 and PKCα/βII are associated in a common pathway due to in vivo IL-13 gene therapy, joint homogenates from rats with AIA were immunoprecipitated with anti–ERK-1/2, followed by immunoblotting for select proteins. Of note, *p-PKCα/βII was up-regulated in the AxCAIL-13 group as compared with the PBS and AxCANI groups (Figure 3D), whereas no association was observed with *p-Tyk2 and *pSTAT6 (results not shown). These data indicate a novel signaling pathway involving ERK-1/2 and PKCα/βII in IL-13–mediated antiangiogenesis in vivo.

In order to confirm the in vivo findings and test whether IL-13 similarly induces phosphorylation of Tyk2, ERK-1/2, and PKCα/βII, as observed in the joint homogenates from the rats treated with AxCAIL-13, U937 cells were treated with various concentrations of human IL-13. Western blot analysis showed that IL-13 induces the activation of *p-Tyk2, *p–ERK-1/2, and *p-PKCα/βII in a concentration-dependent manner (Figure 3E). These results were similar to the expression profiles of the same proteins observed in the joint.

Altered expression of pro- and antiangiogenic mediators in vivo following preventative IL-13 gene therapy. To determine if IL-13 gene delivery indirectly mediates its angiostatic effects through anti- or proangiogenic mediators, we analyzed by ELISA the protein expression of select cytokines, chemokines, and growth factors in joint homogenates obtained on day 18 from rats with AIA (Figure 4). LIX/CXCL5, the rat homolog of angiogenic epithelial neutrophil–activating peptide 78 (ENA-78)/CXCL5, was strongly down-regulated by AxCAIL-13, implying that this chemokine may contribute to mediating the proangiogenic effects. Similarly, levels of CINC-1/CXCL1, the rat homolog of angiogenic growth-related oncogene α (GROα)/CXCL1, were significantly lower in the AxCAIL-13 group (Figures 4A and B). In addition, IL-18, a cytokine with strong proangiogenic properties, was down-regulated in response to IL-13 gene therapy, an effect that was significant as compared with AxCANI, while

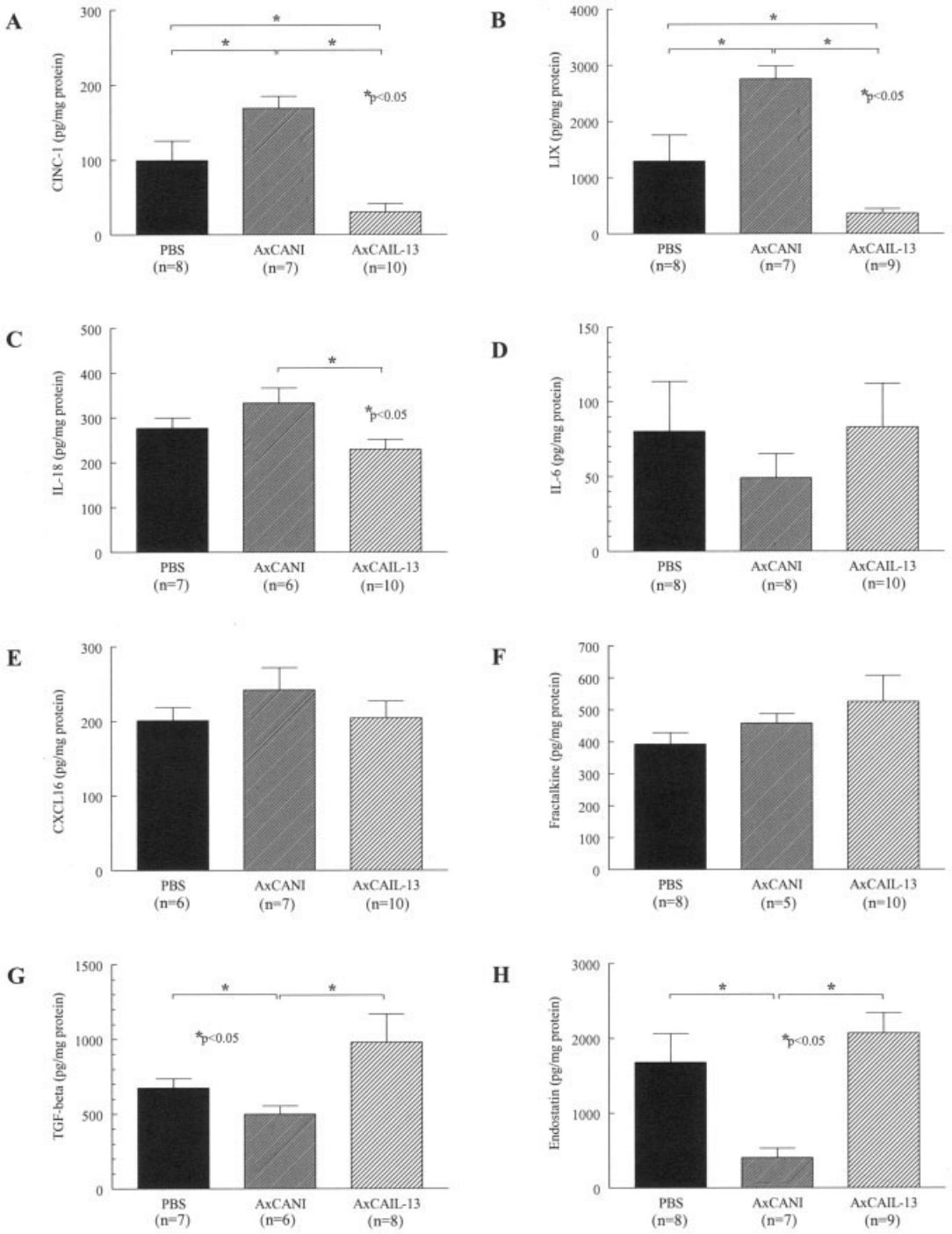

Figure 4. Altered balance of pro- and antiangiogenic mediators following interleukin-13 (IL-13) gene therapy. **A** and **B,** Expression of lipopolysaccharide-induced CXC chemokine (LIX)/CXCL5 and cytokine-induced neutrophil chemoattractant 1 (CINC-1)/CXCL1, the rat homologs of epithelial neutrophil–activating peptide 78/CXCL5 and growth-related oncogene α/CXCL1, respectively, was significantly lower in the AxCAIL-13 group, although AxCANI alone increased the levels of both chemokines as compared with phosphate buffered saline (PBS). **C** and **D,** IL-18, a cytokine with strong proangiogenic properties, but not IL-6, was significantly down-regulated by AxCAIL-13. **E** and **F,** Expression of the proangiogenic membrane-associated chemokines CXCL16 and fractalkine was not affected by AxCANI or AxCAIL-13. **G** and **H,** AxCAIL-13 induced the expression of transforming growth factor β (TGFβ), but also resulted in significantly higher endostatin levels, as compared with AxCANI. Values are the mean and SEM (n = number of joint homogenates).

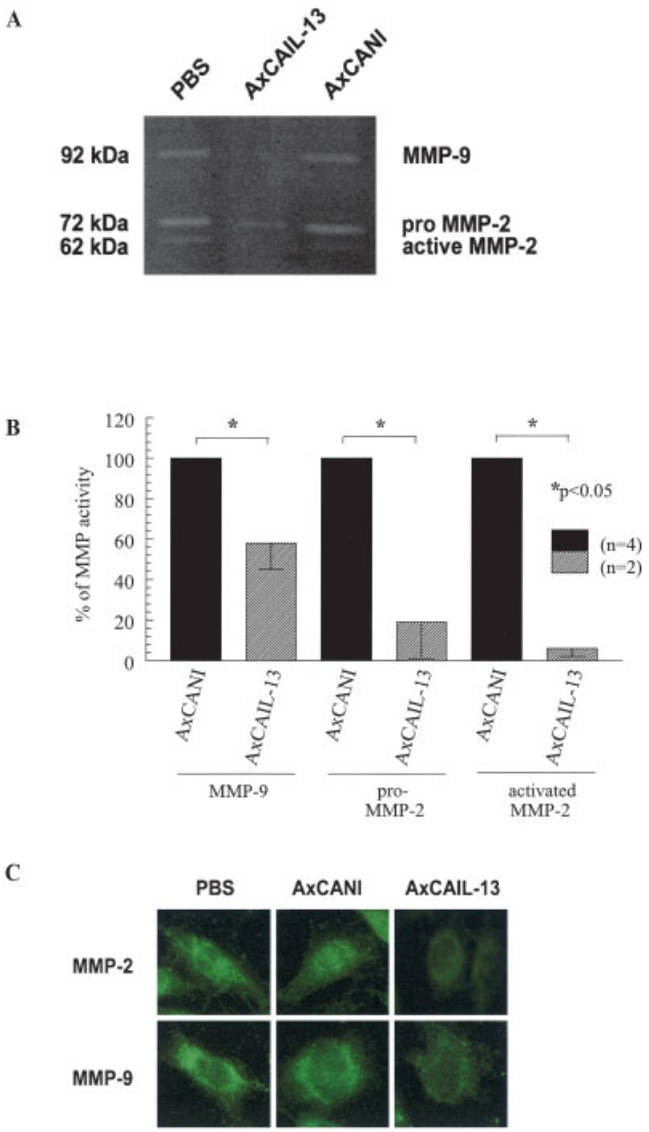

Figure 5. Down-regulation of matrix metalloproteinase 2 (MMP-2) and MMP-9 activity in vivo following interleukin-13 (IL-13) gene therapy. **A,** Zymography showing down-regulation of proMMP-2 (72 kd), active MMP-2 (62 kd), and MMP-9 (92 kd) in AxCAIL-13–treated joint homogenates from rats with adjuvant-induced arthritis (AIA). **B,** Quantification of gelatinase activity as determined by densitometry, confirming the reduction of MMP-2 and MMP-9 levels following AxCAIL-13 treatment. Gelatinase activity of both AxCANI– and phosphate buffered saline (PBS)–treated homogenates was comparable (data not shown). Values are the mean and SEM (n = number of joint homogenates). **C,** Immunofluorescence staining of human dermal microvascular endothelial cells stimulated with joint homogenates from rats with AIA, showing somewhat decreased expression of MMP-2 and MMP-9 proteins in the AxCAIL-13 group. IgG served as negative control. Photomicrographs are representative of 2 independent experiments (original magnification ×400).

the expression of IL-6, which is possibly proangiogenic (32), was not affected (Figures 4C and D).

Levels of the membrane-bound chemokines CXCL16 and fractalkine, both of which are known to promote neovascularization, were not altered by AxCAIL-13 (Figures 4E and F). TGFβ, a bifunctional regulator of angiogenesis, was up-regulated, an effect that was significant compared with AxCANI (Figure 4G). Expression of the angiogenesis inhibitor endostatin was also significantly increased in AxCAIL-13–treated joint homogenates as compared with the vector control AxCANI, whereas application of the adenovirus vector without the insert resulted in a down-regulation of endostatin expression (Figure 4H).

Down-regulation of MMP-2 and MMP-9 by in vivo IL-13 gene delivery. To investigate whether IL-13 may exert its in vivo angiostatic effects in part via MMP-2 and MMP-9, joint homogenates from PBS-, AxCANI-, and AxCAIL-13–treated rats with AIA were analyzed by gelatin zymography (Figure 5). The gelatinase activity was clearly down-regulated by IL-13 gene therapy as compared with AxCANI (Figures 5A and B), whereas it was comparable in both the AxCANI and the PBS group (data not shown). Protein expression of MMP-2 and MMP-9 on HMVECs stimulated with rat AIA homogenates showed similar results, with intracellular down-regulation of both gelatinases (Figure 5C).

DISCUSSION

Neovascularization is a hallmark of various diseases and a characteristic feature of the joint synovial tissue in RA and is critical for disease progression (30). AIA in the rat is an inflammatory disease associated with neovascularization and has been used to study angiogenesis (33,34). Gene therapy presents an appealing approach to the study of the pathophysiologic mechanisms in vivo and has been proven to be useful in studying angiogenesis in various animal models (35–39). In addition, it has been shown that genes encoding antiarthritic properties can be transferred to specific in vivo sites and alter inflammatory responses (40).

Several genes showing promise for use in gene therapy for RA include IL-4, IL-1 receptor antagonist protein, IL-1, TNF soluble receptors, TGFβ, and IL-13, among others (22,40). With regard to IL-13 expression, we and other investigators have shown that IL-13 directly down-regulates arthritis and inflammatory responses in the joints of rodent models of RA (7,41). In addition, we previously showed that IL-13 expression attenuated MMP-mediated cartilage degradation in an

immune complex model of RA, explaining in part, the antiinflammatory properties of IL-13 (42). In the current study, we investigated the possibility that IL-13 also functions as an antiangiogenic mediator in the arthritic joint.

In an earlier study, we demonstrated that IL-13 delivered by gene therapy into the joints of rats with AIA results in decreased synovial blood vessel numbers (7). However, it was unclear if IL-13 directly inhibits the de novo growth of blood vessels in the arthritic joint, or whether it takes on an indirect role by down-regulating angiogenic mediators. For instance, Bessis et al (43) previously showed that IL-13 decreases the expression of human TNFα in a transgenic murine model. Their findings suggest that IL-13 acts in part to modulate angiogenesis by inhibiting the production of proangiogenic cytokines. Interestingly, in vitro analysis of IL-13 has shown it to exert both pro- and antiangiogenic properties dose-dependently (12,13). To clarify these issues, we evaluated the effect of IL-13 gene therapy on neovascularization in vivo, using rat AIA as a model of pathologic blood vessel growth and joint inflammation.

We initially determined hemoglobin levels in the joints of rats with AIA as a reflection of the density of functional blood vessels in the synovium. Decreased hemoglobin content in response to AxCAIL-13 is suggestive of antiangiogenic properties of IL-13 in vivo (Figure 1A). First, we showed in vitro that rat AIA joint homogenate–induced EC migration was significantly inhibited by AxCAIL-13 (Figure 1B), suggesting a net angiostatic effect of in vivo IL-13 gene delivery. We used day 18 AxCAIL-13 homogenates, since these have previously been shown to express elevated levels of IL-13 for at least 10 days after delivery (7), and we compared them with day 18 AxCANI homogenates. Because EC migration was not affected by AxCANI, the antimigratory role seems to be specific for IL-13. Second, we determined that EC tube formation in an in vitro capillary morphogenesis assay was not seen with AxCAIL-13 (Figure 1C), suggesting that IL-13 inhibits EC differentiation and results in less blood vessel growth in vivo. Third, we confirmed these findings in the rat aortic ring assay (Figures 2A and B), in which both EC migration and differentiation take place. While significant sprouting occurred in response to joint homogenates from the PBS and AxCANI groups, IL-13 gene delivery resulted in significantly less sprout formation. Fourth, to determine if IL-13 exerts its angiostatic role in vivo, we tested the ability of rat AIA homogenates to induce blood vessel penetration and formation in an in vivo Matrigel plug angiogenesis assay (Figure 2C). In vivo blood vessel growth was significantly inhibited in response to AxCAIL-13 homogenates, which corroborated previous histologic data (7).

These results provide direct evidence that IL-13 is able to inhibit important steps in neovascularization, namely, EC migration and differentiation, as well as blood vessel formation, in vivo.

Next, using whole joint homogenates, we identified signal transduction pathways associated with IL-13–mediated inhibition of inflammation and angiogenesis (29,44) (Figure 3 and Table 1). The MAPK pathway has been implicated in IL-13–induced signaling via IRS-1 and IRS-2 (45), and we hypothesized that signaling cross-talk among MAPKs and other signaling molecules may play an important role in IL-13–mediated angiostatic effects. We observed a strong phosphorylation of ERK-1/2, but not p38 MAPK or JNK1/2. Up-regulation of *p–ERK-1/2 was independent of the JAK/STAT6 pathway. However, using immunoprecipitation, we discovered an association between ERK-1/2 and PKCα/βII in response to IL-13 gene therapy, pointing to a novel mechanism of IL-13 signaling. Up-regulation of *p-Raf supports the notion that the ERK-1/2 pathway is activated by IL-13 in vivo.

The biologic function of MAPK pathways in this context, however, remains unclear. Only very recently has ERK-1/2 been shown to mediate IL-13–induced lung inflammation and remodeling in vivo (11). Consistent with our results, the selective stimulation of ERK-1/2 was found to be independent of STAT6 (11). In the current study, we showed that *p–NF-κB p65 was down-regulated by AxCAIL-13. This is of importance since NF-κB not only has proinflammatory properties (46), but its activation can also mediate angiogenesis, for example, by up-regulating proangiogenic cytokines, growth factors, and MMPs (47). Thus, decreased expression of *p–NF-κB p65 may promote inhibition of neovascularization. Our findings further suggest that IL-13 may exert its antiangiogenic function in vivo via activation of PKCα/βII and ERK-1/2, with concomitant down-regulation of the NF-κB p65 pathway (Figure 6).

To determine whether IL-13 treatment changes the balance between pro- and antiinflammatory factors in vivo, we determined protein expression of select cytokines in the homogenates (Figure 4). LIX/CXCL5, the rat homolog of the proangiogenic chemokine ENA-78/CXCL5, was strongly down-regulated by AxCAIL-13. This is consistent with our previous data, showing that ENA-78/CXCL5 accounted for a significant proportion of the chemotactic activity for ECs as well as angiogenic activity in RA synovial tissue homogenates (48). Like-

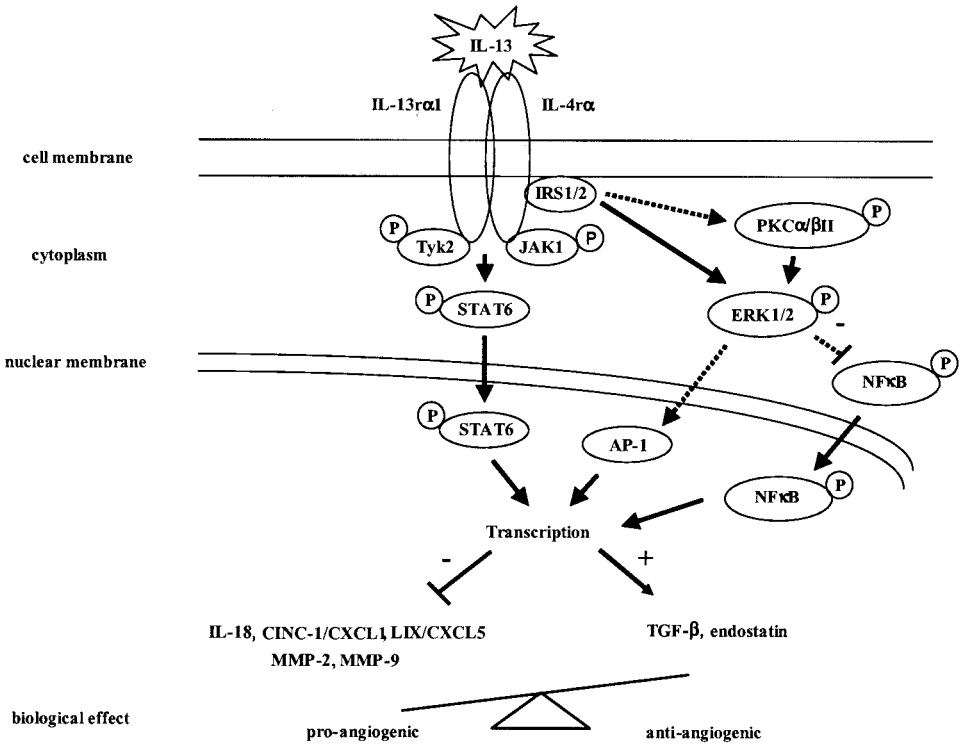

Figure 6. Schematic model of interleukin-13 (IL-13)–induced angiostatic effects in vivo. IL-13 activates insulin receptor substrate 1/2 (IRS-1/2), which in turn, activates ERK-1/2, either directly or via protein kinase Cα/βII (PKCα/βII). ERK-1/2 phosphorylation (P) possibly down-regulates NF-κB phosphorylation and impairs subsequent translocation to the nucleus, thereby governing the transcription of inducers and inhibitors of angiogenesis. Alternatively, ERK-1/2 may act via other transcription factors known to be downstream, such as activator protein 1 (AP-1). Another possibility is that IL-13 signals via the JAK/STAT6 pathway, initially activating Tyk2 and JAK1. STAT6 translocation possibly also shifts the balance between pro- and antiangiogenic factors. Solid arrows represent known mechanisms; broken arrows represent hypothesized mechanisms. IL-13rα1 = IL-13 receptor α1; CINC-1 = cytokine-induced neutrophil chemoattractant 1; LIX = lipopolysaccharide-induced CXC chemokine; MMP-2 = matrix metalloproteinase 2; TGFβ = transforming growth factor β.

wise, levels of CINC-1/CXCL1 were significantly lower in the AxCAIL-13 group, which corroborates the ability of the human homologs of CINC-1, GROα/CXCL1, to promote angiogenesis (49). IL-18, a proangiogenic cytokine (23), was significantly down-regulated in response to IL-13 gene therapy in vivo, while there was no change in the expression of either IL-6 or the potent angiogenic chemokines CXCL16 and fractalkine/CX_3CL1 (50,51). Increased expression of TGFβ, which is known to play a dual role in neovascularization (52,53), suggests an antiangiogenic role in response to IL-13, possibly via the activin receptor–like kinase 5/Smad2/3 pathway (54). In contrast, endostatin, a powerful angiogenesis inhibitor in vitro and in vivo (55), was up-regulated as compared with the vector control.

Since gelatinases are important regulators and modifiers of angiogenesis, we determined whether IL-13 gene therapy altered MMP-2 and MMP-9 expression and activity (Figure 5). Reduced expression and gelatinolytic activity of both MMPs in rat AIA joint homogenates in response to IL-13 pointed to another possible angiostatic mechanism. This is in contrast to MMP-2 and MMP-9 up-regulation by IL-13 in the murine lung that has been described (56). However, IL-13–associated effects might be differentially modulated in this type 2–polarized environment or in the context of angiogenesis.

In conclusion, we have shown that 1) IL-13 gene therapy inhibits angiogenesis in arthritis in vivo, 2) IL-13 activates a novel signaling pathway via PKCα/βII and ERK-1/2 in vivo, 3) IL-13–mediated angiostatic effects in vivo are associated with an altered balance of pro- and antiangiogenic cytokines, and 4) IL-13 may exert its

antiangiogenic effects in part by down-regulating MMP-2 and MMP-9. This study shows that IL-13 gene therapy is a useful approach to the reduction of neovascularization in arthritis in vivo. Knowledge of the specific angiostatic role of IL-13 may help to optimize therapeutic opportunities in the targeting of pathologic blood vessel growth, a condition central to the pathology of RA.

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AUTHOR CONTRIBUTIONS

Drs. Koch, Haas, Amin, and Ruth had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Haas, Amin, Ruth, Allen, Ahmed, Pakozdi, Woods, Koch.

Acquisition of data. Haas, Amin, Ruth, Allen, Ahmed, Pakozdi, Woods, Shahrara.

Analysis and interpretation of data. Haas, Amin, Ruth, Ahmed, Pakozdi, Koch.

Manuscript preparation. Haas, Ruth, Koch.

Statistical analysis. Haas, Ruth.

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