Inhibition of Angiogenesis by Interleukin-4 Gene Therapy in Rat Adjuvant-Induced Arthritis

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Objective. **Interleukin-4 (IL-4) can modulate neovascularization. In this study, we used a gene therapy approach to investigate the role of IL-4 in angiogenesis in rat adjuvant-induced arthritis (AIA), a model for rheumatoid arthritis.**

Methods. **Rats received an adenovirus producing IL-4 (AxCAIL-4), a control virus without insert, or control vehicle (phosphate buffered saline) intraarticularly before arthritis onset. At peak onset of arthritis, rats were killed. Vascularization was determined in the synovial tissue, and correlations with inflammation were assessed. Ankle homogenates were used in angiogenesis assays in vitro and in vivo, and protein levels of cytokines and growth factors were assessed by enzyme-linked immunosorbent assay. Synovial tissue expression of αv integrins was determined by immunohistochemistry.**

Results. **IL-4 induced a reduction in synovial tissue vessel density, which was paralleled by a decrease in inflammation. AxCAIL-4 joint homogenates significantly ($P < 0.05$) inhibited both endothelial cell (EC) migration and tube formation in vitro. Similarly,**

AxCAIL-4 inhibited capillary sprouting in the rat aortic ring assay, and vessel growth in the in vivo Matrigel plug assay. The angiostatic effect occurred despite high levels of vascular endothelial growth factor (VEGF), and was associated with down-regulation of the proangiogenic cytokines IL-18, CXCL16, and CXCL5 and up-regulation of the angiogenesis inhibitor endostatin. Of interest, AxCAIL-4 also resulted in decreased EC expression of the αv and β3 integrin chains.

Conclusion. **In rat AIA, IL-4 reduces synovial tissue vascularization via angiostatic effects, mediates inhibition of angiogenesis via an association with altered pro- and antiangiogenic cytokines, and may inhibit VEGF-mediated angiogenesis and exert its angiostatic role in part via αvβ3 integrin. This knowledge of the specific angiostatic effects of IL-4 may help optimize target-oriented treatment of inflammatory arthritis.**

Interleukin-4 (IL-4), a pleiotropic cytokine and crucial modulator of the immune system (1), has been implicated in neovascularization (2–4). However, its specific role and mechanisms in the context of angiogenesis remain unclear. IL-4 has been demonstrated to have inhibitory effects on the critical steps of angiogenesis in vitro (3), a role that has been supported by in vivo studies showing that tumors expressing IL-4 have reduced vascularity (4). Similarly, we have shown that IL-4 blocks induction of basic fibroblast growth factor (bFGF)–mediated corneal neovascularization in rats (2). In contrast, other studies have shown that IL-4 may induce angiogenesis in vitro and in vivo (5,6). Of note, the pro- or antiangiogenic role may be dose-dependent (2).

Inflammatory synovitis is a hallmark feature of rheumatoid arthritis (RA) and strongly associated with neovascularization in the synovial tissue (7). The process of neovascularization is of pivotal importance in the progression of the disease, by creating a direct conduit for the influx of inflammatory cells and thereby exacer-

bating the inflammatory response (8). Thus, targeting neovascularization is an interesting and promising approach for the treatment of RA.

IL-4 cannot be detected in the serum or synovial fluid of RA patients (9,10), while studies in animal models of arthritis have yielded conflicting results (11,12). For example, administration of IL-4 ameliorated the course of murine streptococcal cell wall–induced arthritis (13) and significantly reduced disease incidence and severity in murine collagen-induced arthritis (CIA) (14). In contrast, Lubberts et al noted enhanced onset and aggravation of synovial inflammation following IL-4 treatment in CIA (15), and in a recent study using the K/BxN model, IL-4 promoted arthritis (11). We recently demonstrated that IL-4, preventatively delivered by adenoviral gene therapy, improved arthritis and attenuated inflammation and vascularization of the synovium in rat adjuvant-induced arthritis (AIA) (12). Our experiments revealed a dramatic clinical improvement following preventative administration of IL-4; nevertheless, the treatment failed to lower levels of crucial proinflammatory cytokines, such as tumor necrosis factor α (TNFα) or IL-1β, suggesting that modulation of other factors may be occurring.

Important mediators of angiogenesis include integrins, which are transmembrane glycoproteins consisting of 2 non–covalently bound α- and β-chains (16). Integrins $\alpha v\beta3$ and $\alpha v\beta5$ can clearly modulate angiogenesis and have become a focus in strategies aimed at modifying neovascularization (16). In a rabbit model of arthritis, injection of an $\alpha v\beta3$ antagonist not only inhibited angiogenesis but also attenuated disease manifestations (17). Of note, expression of αv integrin can be modified by cytokines, including IL-4 (18).

In this study, we show that reduced synovial tissue vascularization in rat AIA in response to preventative IL-4 gene therapy could be attributed to inhibition of angiogenesis. This effect was associated with a significant shift in the balance of pro- and antiangiogenic factors in vivo, but also could be mediated directly via IL-4. We also provide evidence that the angiostatic response may be mediated, in part, by altered expression of $\alpha v\beta3$ integrin.

MATERIALS AND METHODS

Preparation, propagation, purification, and titering of adenoviruses. Replication incompetent adenoviruses that produce rat IL-4 (AxCAIL-4) or having no foreign gene insert (AxCANI) were prepared via homologous recombination in 293 cells, as described previously (12). In brief, expression of the rat IL-4 gene was directed by the chicken β-actin promoter and the cytomegalovirus enhancer of pAxCAwt, a 45-kb cosmid containing the full-length sequence of type 5 adenovirus deleted of the E1A, E1B, and E3 regions (19,20). Viruses were propagated in 293 cells, purified, and titered as described previously (21).

Induction of rat AIA, experimental setup, and tissue sampling. Female Lewis rats (weight 100 grams) were injected subcutaneously with 300 μl (5 mg/ml) lyophilized *Mycobacterium butyricum* (Difco, Detroit, MI) in sterile mineral oil, administered at the base of the tail on day 0. Rats were divided into 3 groups, comprising an AxCAIL-4 group as well as 2 control groups, one designed to receive phosphate buffered saline (PBS) and the other to receive AxCANI (virus without insert); the latter group was included to distinguish between the adenoviral and IL-4–mediated effects, since use of adenoviruses in animal models of arthritis has been shown to result in increased inflammation (12,15). In this preventative treatment design, 5×10^8 plaque-forming units of AxCAIL-4 or AxCANI, or PBS, was administered intraarticularly into each ankle on day 8 postinduction, prior to development of arthritis. All animals were killed at day 18 after adjuvant injection, at the time of maximal inflammation, and rat joints were collected for further examination.

Ankle homogenates and protein extracts. For enzyme-linked immunosorbent assays (ELISAs) and angiogenesis assays, rat joints were skinned, weighed, and frozen at −80°C. Ankles were homogenized as described previously (12), and the concentration of protein in each lysate was determined by using a bicinchoninic acid assay (Pierce, Rockford, IL). Hemoglobin content was determined by the tetramethylbenzidine (TMB) method, and compared with a standard curve.

Cell culture. Human dermal microvascular endothelial cells (HMVECs) (Cambrex, Walkersville, MD) were maintained in growth factor–complete endothelial cell (EC) basal medium (EBM) supplemented with 10% fetal bovine serum (FBS) and additional growth factors. Cells were maintained at 37°C in 5% CO_2 and used between passages 5 and 12.

HMVEC chemotaxis assay. HMVEC chemotaxis was performed using a 48-well Boyden chemotaxis chamber (Neuroprobe, Cabin John, MD) as described previously (22). HMVECs (4×10^4 cells/well in EBM + 0.1% FBS) were plated in the bottom wells of the chambers with a polyvinylpyrrolidone-free polycarbonate filter (8 μm pore size; Nucleopore, Pleasant, CA). Chambers were inverted and incubated at 37°C in 5% CO_2 for 2 hours, which allowed HMVECs to attach to the membrane. The chambers were inverted again, and pooled joint homogenates from 5 rat ankles per group (60 μg protein per ml) or PBS or positive control bFGF (60 nM) were added. The chambers were incubated at 37°C for 2 hours. To determine the role of proangiogenic factors in rat AIA joints upon HMVEC migration, pooled homogenates of AxCANI-treated ankle joints were preincubated (37°C for 60 minutes) with neutralizing goat anti–IL-18 or goat anti-CXCL16 antibodies (10 μg/ml; R&D Systems, Minneapolis, MN), prior to being used as stimulants. AxCANI and AxCAIL-4 homogenates preincubated with goat IgG served as controls. Chemotaxis was performed in quadruplicate, with results expressed as the number of migrated

HMVECs per 3 high-power fields (hpf) (at 400× magnification). Each assay was repeated at least 3 times.

In vitro capillary morphogenesis assay. Assessment of tube formation by HMVECs in growth factor–reduced (GFR) Matrigel was used to evaluate the effect of AxCAIL-4 rat joint homogenates on capillary morphogenesis, as described previously (23). HMVECs were suspended in EBM with 1% FBS and seeded in Labtek chamber slides on GFR Matrigel (Becton Dickinson, Bedford, MA) at a density of 1.6×10^4 cells per chamber. Immediately after plating, pooled rat joint homogenates (from 5 rat ankles per group; 60 μg protein per ml) from the AxCAIL-4–, AxCANI-, and PBS-treated groups, phorbol myristate acetate (PMA) (50 nM) as positive control, or DMSO in place of PMA as vehicle control were added to the cell suspension. After 16–18 hours of incubation at 37°C in 5% CO_2, capillary morphogenesis was examined under a phase-contrast microscope. Node formation (defined as a nodular contact formation of at least 3 adherent EC tubes) was determined in a blinded manner, and the number of circular tube network formations was evaluated. Similarly, EC tube formation in response to AxCAIL-4 was determined in the presence of neutralizing antibodies to rat IL-4 (BD PharMingen, San Diego, CA), human CXCL16, and human IL-18 (R&D Systems). Each assay was performed 3 times.

Rat aortic ring sprouting growth assay. Aortas were removed from female Sprague-Dawley rats (weight 200 grams), cleaned of surrounding connective tissue, and sliced into 1-mm–thick rings (24). Aortic rings were then placed into 300 μl of Matrigel in 48-well plates. Serum-free EBM (300 μl) containing rat joint homogenates from 8 rats per group (60 μg protein per ml) was added. Basic FGF (100 nM) and PMA (50 nM) were used as positive controls, while PBS and DMSO served as negative vector controls. Aortic rings were incubated at 37°C in 5% CO_2 to allow microvessel sprouting from the adventitial layer. Sprouting was measured using the following scale: 0 = no sprouting; 1 = migrated cells without sprouting; 2 = isolated sprouting; 3 = sprouting in 25–50% of the arterial ring circumference; 4 = sprouting in 50–75% of the circumference; and 5 = sprouting in 75–100% of the circumference.

Matrigel plug in vivo angiogenesis assay. To examine the effects of AxCAIL-4 treatment on angiogenesis in vivo, Matrigel plug assays were performed as described previously (25). C57BL/6 mice (10–11 mice per group; NCI, Bethesda, MD) were anesthetized by isofluorane inhalation (Abbott Pharmaceuticals, Abbott Park, IL), shaved on the ventral aspect, and given a subcutaneous injection of GFR Matrigel (500 μl/injection) containing pooled rat joint homogenates (from 5 rats) at a final concentration of 120 μg protein per ml. Matrigel containing PBS or bFGF (1 ng/ml) served as a negative and positive control, respectively. After 7 days, plugs were dissected and homogenized, and hemoglobin content was determined as described above.

ELISAs. Six-to-eight ankle homogenates were analyzed using commercially available ELISA kits for the following rat cytokines and growth factors: cytokine-induced neutrophil chemoattractant 1 (CINC-1)/CXCL1, lipopolysaccharide-induced CXC chemokine (LIX/CXCL5), IL-6, IL-13, IL-18, transforming growth factor β (TGFβ), vascular endothelial growth factor (VEGF), and endostatin. ELISAs were performed according to the manufacturer's protocol. CXCL16 levels were measured by coating 96-well polystyrene plates with rabbit anti-human CXCL16 (PeproTech, Rocky Hill, NJ),

followed by a blocking step as described previously (26). Biotinylated rabbit anti-human antibody (PeproTech) was used to detect CXCL16 with the streptavidin–peroxidase method (BD PharMingen) and TMB as color substrate (Sigma, St. Louis, MO). All samples were analyzed in duplicate.

Immunohistochemistry. Immunohistochemistry was performed on 8 μm synovial tissue rat AIA cryosections (3–5 rats per group) for the EC marker von Willebrand factor (vWF) (DakoCytomation, Carpinteria, CA) and the integrin chains αv, β3, and β5 (rabbit anti-rat antibodies; Santa Cruz Biotechnology, Santa Cruz, CA). Isotype-matched nonspecific IgG was used as a negative control. Immunostaining was performed using Vector Elite ABC Kits (Vector, Burlingame, CA) and diaminobenzidine (Kirkegaard & Perry, Gaithersburg, MD) as a chromogen, followed by counterstaining with hematoxylin.

Microscopic analysis. Various synovial tissue cell types were evaluated for positive staining, and included lining cells, mononuclear cells (MNCs), ECs, and smooth muscle cells (SMCs). Immunostaining was evaluated and graded by a pathologist (GKH III) in a blinded manner, as described previously (27). Cell types were distinguished on the basis of their morphologic characteristics and/or immunohistochemical staining reaction, as previously described (26,28). Each synovial tissue component was graded using a frequency of staining scale, ranging 0–100%, in which 0% indicates no staining and 100% indicates that all cells are immunoreactive. The percentage of reactivity was calculated as the number of cells of a given type reacting with a specific antibody divided by the total number of cells of that given type. Synovial tissue vascularity was scored on a scale of 1–4, as follows: 1 = marked decrease in vessels; 2 = normal density in vessels; 3 = increased density in vessels; and 4 = marked increase in vessel density, resembling granulation tissue. In addition, an inflammation score was obtained using the following scoring system: 1 = normal; 2 = increased number of inflammatory cells, arrayed as individual cells; 3 = increased number of inflammatory cells, including distinct clusters (aggregates); and 4 = marked diffuse infiltrate of inflammatory cells. Score data were pooled and expressed as the mean $\pm$ SEM of each data group.

Statistical analysis. All values are presented as the mean $\pm$ SEM. Since rats received identical injections in each ankle based on their group assignment, and rats with AIA often developed inflammation to different degrees in each of the hind limbs, each ankle was assessed independently for statistical purposes (12). Statistical analysis was performed using Student's t-test, with a P value less than 0.05 considered statistically significant. Pearson's correlation coefficients were assessed to describe the relationship between vascularity score and synovial tissue inflammation.

RESULTS

IL-4–mediated decrease in synovial vascularization paralleled by reduction in inflammation. To study the effect of IL-4 gene therapy on vascularization in the context of inflammatory changes, vessel density was assessed and correlated with the extent of histologic inflammation. Injection of rat ankle joints with AxCANI was associated with an increase in vascularity score, which was significantly reduced by treatment with
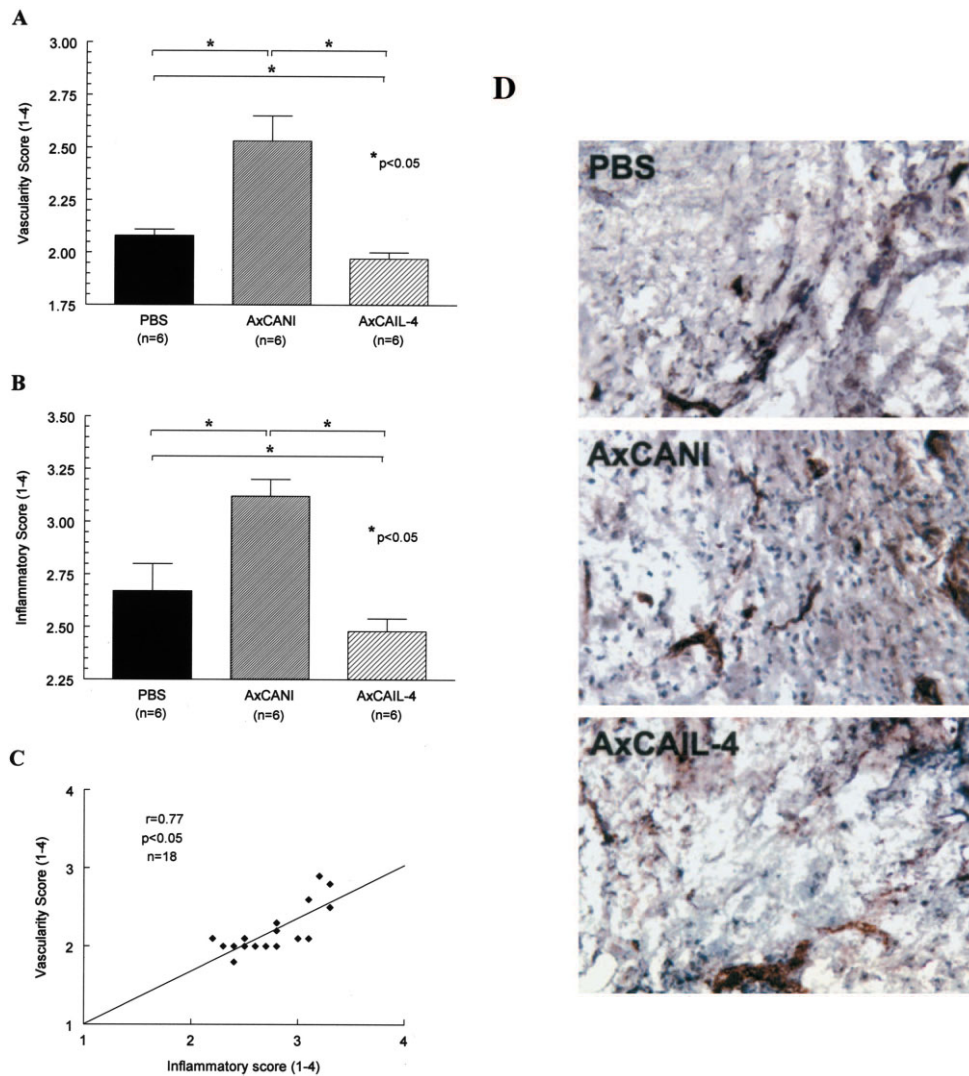
Figure 1. Reduction of synovial neovascularization in rat adjuvant-induced arthritis (AIA) ankle joints by intraarticular injection of interleukin-4–producing adenovirus (AxCAIL-4). **A,** The histologic vascularity score (range 1–4) was determined by assessing the vessel density in the synovial tissue from AxCAIL-4–treated rats with AIA as compared with the group receiving control virus without insert (AxCANI) or phosphate buffered saline (PBS)–treated rats. **B,** The histologic inflammation score (scale 1–4) was assessed by determining the presence of inflammatory cells in the synovium from each group. Bars show the mean and SEM. **C,** The vascularity score of all groups (n = 18) strongly correlated with the extent of inflammation in the rat joints. **D,** Photographs of representative immunohistologic findings for the endothelial cell marker von Willebrand factor, showing reduced synovial vascularity in the AxCAIL-4–treated animals compared with AxCANI-treated synovial tissue (original magnification × 200). Color figure can be viewed in the online issue, which is available at http://www.arthritisrheum.org.

AxCAIL-4, even when compared with the PBS group, suggesting that IL-4 has possible antiangiogenic properties (Figure 1A). Immunostaining with vWF (an EC marker) confirmed decreased vessel density in the AxCAIL-4–treated group (Figure 1D). Of interest, synovial inflammation paralleled the vascularization in the 3 groups, and the inflammation score was significantly reduced in the AxCAIL-4–treated rats compared with the PBS or AxCANI group (Figure 1B). In fact, the vascularity score in all groups was strongly correlated with the extent of inflammation in the rat AIA joints (r = 0.77, $P < 0.05$; n = 18) (Figure 1C).

Significant in vitro inhibition of EC migration by IL-4 gene delivery. To determine whether the increased vascularization in rat AIA joints might be due to an increase in proangiogenic factors, we first tested EC chemotaxis in vitro in response to joint homogenates from PBS-treated animals. The HMVEC migratory re-
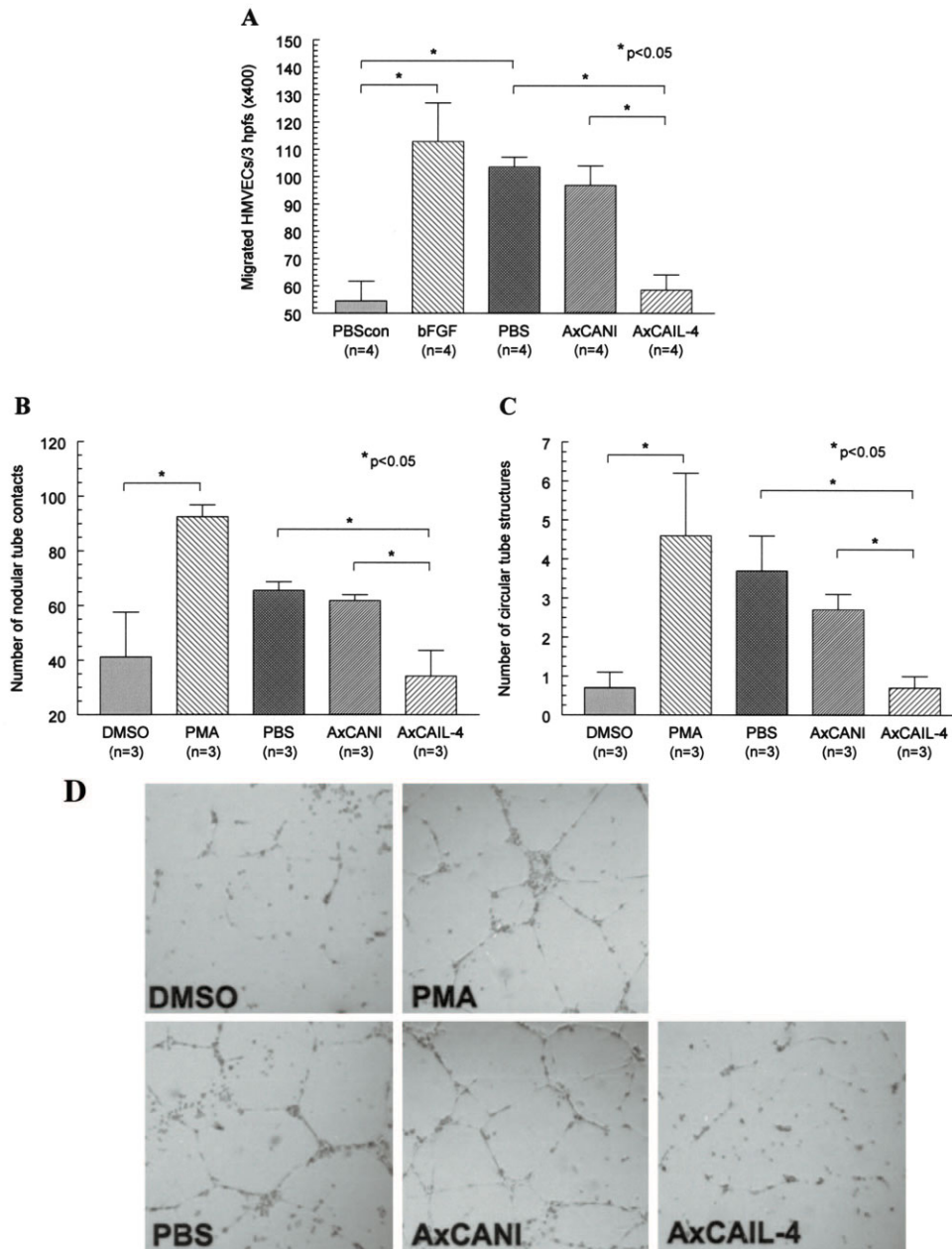
Figure 2. Decrease in rat AIA joint homogenate–induced endothelial cell migration and tube formation in vitro by AxCAIL-4 treatment, as assessed by chemotaxis assay, in vitro capillary morphogenesis assay, and in vitro Matrigel assay using human dermal microvascular endothelial cells (HMVECs) and rat AIA joint homogenates as stimulant. Each assay was performed in triplicate, with results expressed as the mean and SEM of independent experiments. n = number of experiments per group. **A,** In vitro migration of HMVECs in response to joint homogenates (60 μg protein/ml) from each treatment group of rats with AIA compared with PBS as negative stimulant control (PBScon) and basic fibroblast growth factor (bFGF) (60 n*M*) as positive control. **B** and **C,** Endothelial cell tube formation in Matrigel, expressed as the number of nodular tube contacts (**B**) or circular tube structures (**C**) per high-power field (hpf). DMSO served as vehicle control, and phorbol myristate acetate (PMA) as positive control. **D,** Photographs of representative assay findings in each group (original magnification × 200). Color figure can be viewed in the online issue, which is available at http://www.arthritisrheum.org. See Figure 1 for other definitions.

sponse was ~2-fold higher than baseline values and comparable with the effects of bFGF (Figure 2A).

To assess whether the reduction of functional blood vessels in response to IL-4 was due to an angiostatic effect, we further studied EC chemotaxis in vitro in response to joint homogenates from AxCAIL-4– and

AxCANI-treated ankles (Figure 2A). Whereas HMVEC migration in response to AxCANI joint homogenates was comparable with that in the PBS-treated group (mean $\pm$ SEM 96 $\pm$ 7 versus 104 $\pm$ 4 cells/3 hpf), the migratory response was almost completely inhibited by AxCAIL-4 (59 $\pm$ 6 cells/3 hpf; $P < 0.05$), suggesting that IL-4 gene delivery inhibits EC migration, a critical step in the process of neovascularization.

Reduction of EC tube formation by IL-4 gene delivery in the in vitro capillary morphogenesis assay. To elucidate the role of IL-4 gene therapy in another important step of angiogenesis, namely EC differentiation, we studied in vitro EC tube formation in response to PBS, AxCANI, and AxCAIL-4 joint homogenates (Figures 2B, C, and D). Homogenates from PBS- and AxCANI-treated rats with AIA stimulated tube formation. However, AxCAIL-4 treatment resulted in a joint environment that significantly inhibited EC tube formation ($P < 0.05$). These results imply that IL-4 gene delivery attenuates EC differentiation.

Inhibition of rat AIA joint homogenate–induced vessel sprouting by AxCAIL-4 in the rat aortic ring assay. To validate our findings of an inhibitory effect of IL-4 on vessel formation, we tested both EC migration and EC differentiation in the rat aortic ring assay, and examined whether homogenates of AxCAIL-4–treated rat AIA ankle joints were less likely to promote EC sprout formation compared with homogenates derived from the other 2 groups (Figure 3). Sprouting started after ~10 days in the PBS group and showed a constant increase until day 28, thus confirming that rat AIA joint homogenates have proangiogenic properties. AxCANI therapy resulted in similar sprout formation. In contrast, aortic rings treated with AxCAIL-4 joint homogenates did not develop any appreciable sprouting (Figures 3A and B; representative images shown in Figure 3C).

Inhibition of rat AIA joint homogenate–induced angiogenesis in vivo by AxCAIL-4. To test the angiostatic response to IL-4 gene delivery in an in vivo setting, we performed a Matrigel plug in vivo angiogenesis assay. GFR Matrigel containing rat joint homogenates treated with PBS, AxCANI, or AxCAIL-4 were injected subcutaneously into C57BL/6 mice and analyzed after 7 days (Figure 4A). Hemoglobin measurements, as a marker of blood vessel penetration, showed that AIA joint homogenates treated with PBS induced blood vessel formation as compared with the effects of PBS as negative stimulant control (PBScon in Figure 4A) ($P < 0.05$). Joint homogenates from the AxCANI-treated group did not alter hemoglobin content, suggesting that neovascularization was unaffected by the control vector, whereas IL-4 gene delivery resulted in a significant inhibition of
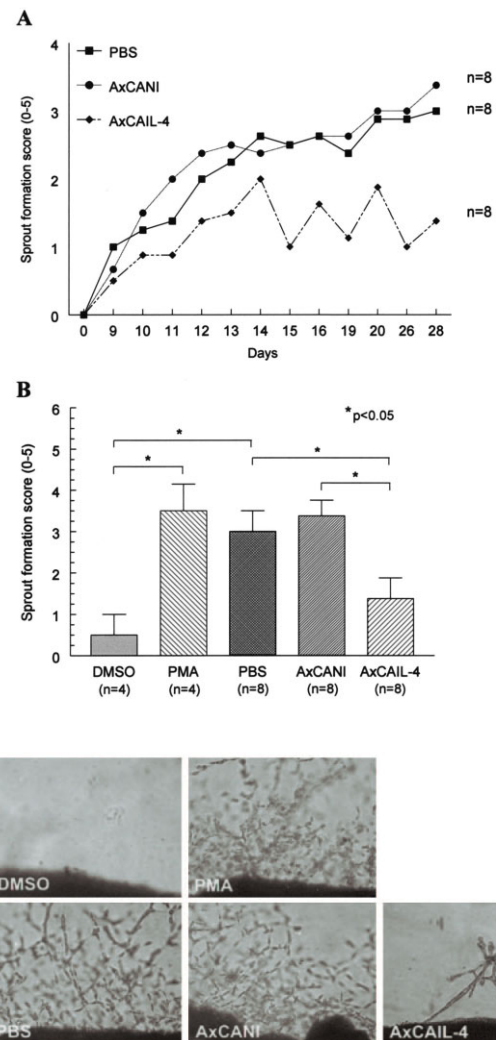

Figure 3. Inhibition of rat AIA joint homogenate–induced vessel sprouting by AxCAIL-4 in the rat aortic ring assay. After placement in growth factor–reduced Matrigel and covering with medium plus rat joint homogenates (at 60 μg/ml protein), aortic rings were observed for vessel sprouting using a scale of 0–5. **A,** Sprout formation score over time in the 3 treatment groups (n = 8 joint homogenates per group). **B,** Sprout formation score at day 28 in response to treated rat AIA joint homogenates compared with phorbol myristate acetate (PMA)–induced sprouting and compared with DMSO vehicle control. Bars show the mean and SEM. **C,** Photographs of representative aortic ring sprouting in each group (original magnification $\times$ 200). Color figure can be viewed in the online issue, which is available at http://www.arthritisrheum.org. See Figure 1 for other definitions.

blood vessel growth ($P < 0.05$), showing that AxCAIL-4 impairs the proangiogenic environment in rat AIA joints.

To confirm these findings, we determined whether IL-4 gene delivery could reduce vascularity (which is proportional to hemoglobin content) in the rat AIA joints. Hemoglobin levels, as measured in the ankle homogenates, increased in response to AxCANI treat-
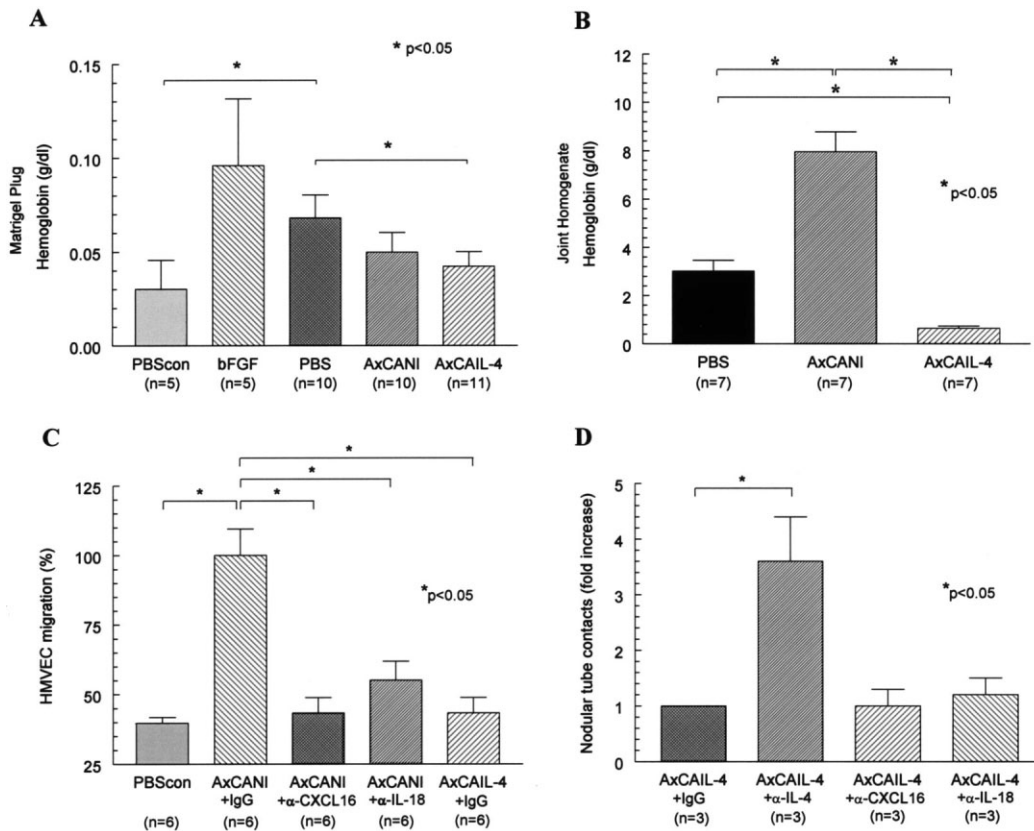
Figure 4. Inhibition of rat adjuvant-induced arthritis (AIA) joint homogenate–induced angiogenesis in vivo mediated directly or indirectly by interleukin-4 (IL-4)–producing adenovirus (AxCAIL-4). **A,** Assessment of blood vessel growth, reflected in hemoglobin content, in response to treated rat AIA joint homogenates, using Matrigel plugs with test substance injected into C57BL/6 mice; phosphate buffered saline (PBS) served as negative stimulant control (PBScon) and basic fibroblast growth factor (bFGF) (1 ng/ml) as positive control. n = number of mice. **B,** Hemoglobin content as an indirect marker for neovascularization in rat joint homogenates, emphasizing the angiostatic effect of IL-4 in vivo. n = number of joint homogenates. **C,** Human dermal microvascular endothelial cell (HMVEC) migration in response to tissue homogenates from rats with AIA treated with control virus without insert (AxCANI) in the presence or absence of neutralizing antibodies. IgG served as control. n = number of assays. **D,** Effects of neutralizing antibodies on AxCAIL-4–induced endothelial tube formation on Matrigel in vitro. IgG served as control. n = number of assays. Bars show the mean and SEM.

ment, but were significantly reduced in the AxCAIL-4–treated group when compared with the PBS- and AxCANI-treated groups ($P < 0.05$) (Figure 4B). Thus, these findings corroborate the observation of IL-4 as an angiostatic cytokine in vivo.

Change in expression of pro- and antiangiogenic mediators using preventative IL-4 gene therapy. To determine whether the angiostatic effects of IL-4 gene delivery were mediated via pro- and antiangiogenic factors, we studied protein expression of select cytokines, chemokines, and growth factors in the rat AIA joint homogenates (Table 1). IL-18 and CXCL16, both of which are cytokines with strong proangiogenic prop-

erties (23,29), were significantly down-regulated following IL-4 gene therapy, whereas the angiogenesis inhibitor endostatin showed up-regulation in the AxCAIL-4–treated group compared with AxCANI-treated animals (mean ± SEM 1,550 ± 266 versus 405 ± 124 pg/mg protein; $P < 0.05$). In addition, expression of LIX/CXCL5, the rat homolog of epithelial-derived neutrophil attractant 78 (ENA-78)/CXCL5, was strongly down-regulated by AxCAIL-4, implying that this chemokine may contribute to mediating the proangiogenic effects. Similarly, levels of CINC-1/CXCL1, the rat homolog of growth-related oncogene α (GROα)/CXCL1, were significantly lower in the AxCAIL-4 group. Expression of IL-6,

Table 1. Pro- and antiangiogenic factors in rat adjuvant-induced arthritis ankle homogenates among the preventatively injected AxCAIL-4, AxCANI, and PBS groups*

Factor, pg/mg protein	PBS	AxCANI	AxCAIL-4
CXCL16	201.0 ± 17.6 (n = 6)	242.1 ± 29.7 (n = 7)	132.3 ± 20.4 (n = 7)†
CINC-1	99.4 ± 26.1 (n = 8)	169.0 ± 16.1 (n = 7)	6.3 ± 3.2 (n = 6)†
LIX	1,296 ± 465 (n = 8)	2,759 ± 229 (n = 7)	180 ± 24 (n = 6)‡
IL-18	276.4 ± 23.2 (n = 7)	332.5 ± 33.9 (n = 6)	162.3 ± 14.4 (n = 8)†
IL-13	66.5 ± 12.8 (n = 8)	32.2 ± 4.7 (n = 6)	31.6 ± 3.6 (n = 7)§
IL-6	80.1 ± 33.6 (n = 8)	49 ± 16.1 (n = 8)	57.2 ± 22.5 (n = 8)
VEGF	35.3 ± 3.8 (n = 7)	36.2 ± 1.2 (n = 6)	54.1 ± 5.1 (n = 6)†
Endostatin	1,678 ± 388 (n = 7)	405 ± 124 (n = 7)	1,550 ± 266 (n = 8)‡
TGFβ	673 ± 64 (n = 7)	499 ± 55 (n = 6)	952 ± 79 (n = 8)†

* Values are the mean ± SEM of the indicated chemokine, cytokine, or growth factor in the phosphate buffered saline (PBS) group and the groups receiving interleukin-4 (IL-4)–producing adenovirus (AxCAIL-4) or control virus without insert (AxCANI). CINC-1 = cytokine-induced neutrophil chemoattractant 1; LIX = lipopolysaccharide-induced CXC chemokine; VEGF = vascular endothelial growth factor; TGFβ = transforming growth factor β.
† $P < 0.05$ versus PBS-injected group and AxCANI-injected group.
‡ $P < 0.05$ versus AxCANI-injected group.
§ $P < 0.05$ versus PBS-injected group.

known to have a proinflammatory role in RA and, possibly, proangiogenic properties (30), was not altered by IL-4 gene delivery, nor was there any effect of AxCAIL-4, when compared with AxCANI, on IL-13, an antiinflammatory cytokine in RA with a dual role in angiogenesis. Surprisingly, VEGF was slightly but significantly up-regulated in rat joints following IL-4 treatment. Expression of TGFβ, known to be a bifunctional regulator of angiogenesis, was also increased by IL-4 gene delivery.

Inhibition of in vitro EC migration by blocking of the proangiogenic cytokines IL-18 and CXCL16. To test whether the observed reduction in proangiogenic factors in response to AxCAIL-4 may explain the angiostatic effects in this study, we studied EC chemotaxis in response to AxCANI joint homogenates following preincubation with neutralizing antibodies (Figure 4C). HMVEC migration was significantly reduced in the presence of anti–IL-18 or anti-CXCL16 antibodies (mean ± SEM 43 ± 6% and 55 ± 7%, respectively, versus 100% in the AxCANI + IgG group; $P < 0.05$) and comparable with the reduction in HMVEC migration in response to AxCAIL-4 homogenates, in which IL-18 and CXCL16 levels were low, suggesting a direct proangiogenic role of IL-18 and CXCL16 in this setting.

Direct antiangiogenic effects of IL-4. Using the in vitro Matrigel assay, we further tested whether the antiangiogenic effects of IL-4 could be mediated directly, rather than indirectly, by altering expression of other cytokines. Blocking of IL-4 using neutralizing antibodies reversed the AxCAIL-4–mediated inhibition of EC tube formation, whereas this was not observed in

the presence of control IgG or antibodies against IL-18 or CXCL16. These results indicate that in addition to reducing the expression of proangiogenic cytokines, IL-4 can also exert a direct effect on target ECs to decrease angiogenesis.

Down-regulation of αv integrins on ECs in response to AxCAIL-4 gene therapy. To determine the in vivo effect of IL-4 on the expression of the αvβ3 and αvβ5 integrins, immunohistochemistry was performed (Figure 5). The integrin αv chain was highly expressed on ECs in PBS-treated rat AIA synovium (mean ± SEM 76.0 ± 6.8%), but was significantly down-regulated by IL-4 (18.3 ± 7.3%; $P < 0.05$). Surprisingly, in response to IL-4, αv integrin expression was increased on synovial MNCs and SMCs ($P < 0.05$), and tended to be higher on lining cells (Figure 5A). Integrin β3 up-regulation on ECs was completely reversed by IL-4 gene delivery ($P < 0.05$). In addition, AxCAIL-4 increased integrin β3 expression on MNCs and lining cells ($P < 0.05$) (Figure 5B). In contrast, synovial expression of the integrin β5 chain was not significantly altered by IL-4 gene delivery (Figure 5C), suggesting that IL-4 exerts its antiangiogenic effects via integrin αvβ3, but not αvβ5.

DISCUSSION

RA is characterized by progressive destruction of the joints, associated with unchecked cellular influx and angiogenesis in the synovial tissue. Proinflammatory cytokines, such as IL-1 and TNFα, have been shown to be pivotal in the pathogenesis and progression of the disease and have been successfully targeted in the treat-
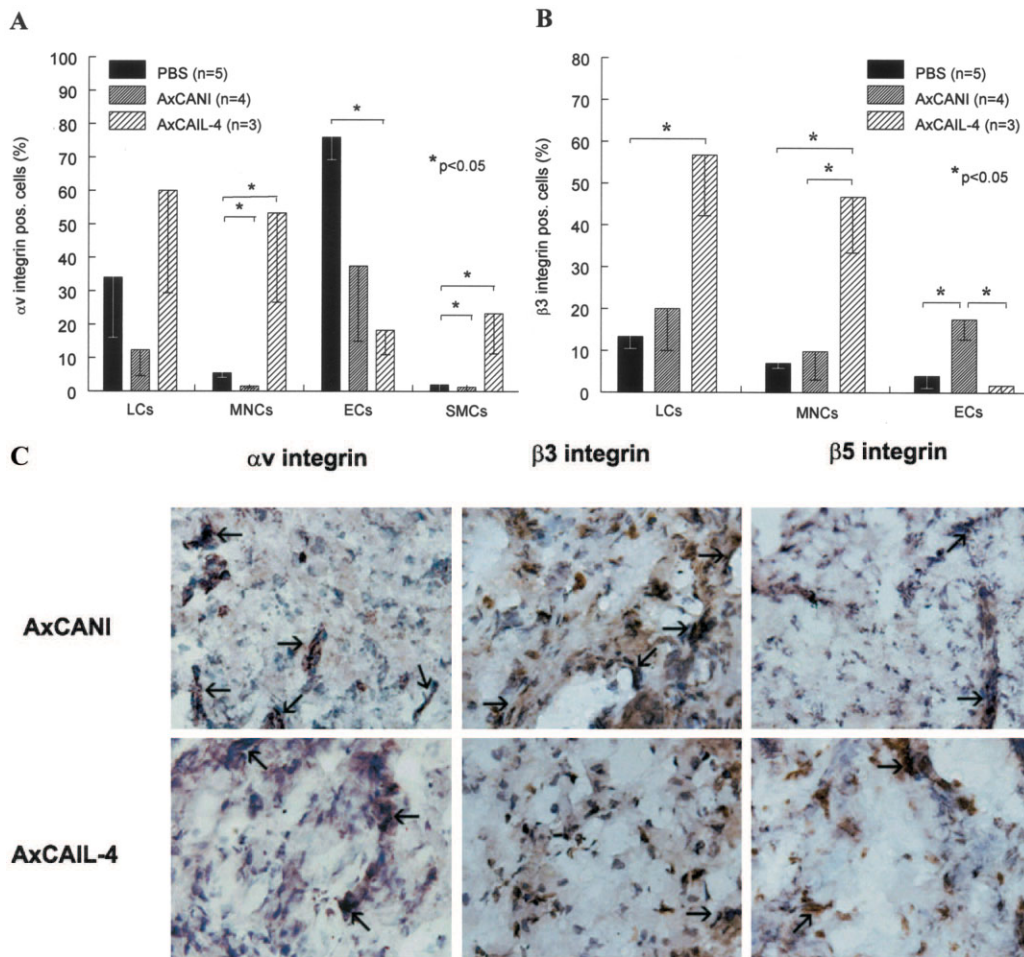
Figure 5. Down-regulation of αv integrins on endothelial cells (ECs) by AxCAIL-4 in rat AIA synovial tissue. Immunohistochemical staining was performed for expression of the integrin chains αv, β3, and β5 on lining cells (LCs), mononuclear cells (MNCs), ECs, and smooth muscle cells (SMCs) in each group. **A** and **B,** The effects of AxCAIL-4 on the integrin αv chain (**A**) and β3 chain (**B**) were compared with those of PBS and AxCANI. **C,** Representative images of staining for integrins αv, β3, and β5 in rat AIA synovial tissue treated with AxCANI or AxCAIL-4 (original magnification × 200). **Arrows** indicate immunopositivity on ECs. n = number of animals. See Figure 4 for other definitions.

ment of RA patients (31). An imbalance of Th1/Th2 cytokines is likely to impact the chronic inflammation associated with RA (32), with IL-4 playing a critical role in the Th2 reaction and able to modulate the IL-1– and TNFα-mediated inflammatory responses (33,34).

Since treatment of RA requires therapeutic measures over a prolonged period, gene therapy has been considered to be an interesting approach and also to have advantages over conventional therapies that use soluble receptors, antagonizing proteins, or blocking antibodies. Indeed, gene therapy has been proven to be useful in various animal models of arthritis (35), and targeting angiogenesis using viral vectors, e.g., for en-

dostatin or soluble VEGF receptor 1, can efficiently modify disease activity (36,37). However, cytokines, such as IL-4, may provide a potential benefit by their ability to also modify the immune response. We have previously shown that IL-4 gene delivery into rat AIA joints resulted in a decreased number of synovial tissue blood vessels (12). However, it is unclear whether IL-4 affects de novo growth of blood vessels in the arthritic joint. Although it is possible that IL-4 has an indirect impact on vessel growth via pro- and antiinflammatory cytokines, it is conceivable that IL-4 treatment inhibits angiogenesis independent of its antiinflammatory properties. In the present study we therefore evaluated the

effect of IL-4 gene therapy on neovascularization in rat AIA and identified possible pathways mediating this process in vivo.

Angiogenesis starts very early in the course of RA and may even precede the specific histopathologic and clinical signs of inflammation (38). In the present study, we show that blood vessel density in the synovium is positively correlated with influx of inflammatory cells, and that both parameters decrease following IL-4 gene delivery (Figure 1). This is consistent with histologic findings in human RA synovium, in which blood vessel proliferation was found only in the presence of inflammation (7,39). Of interest, in human RA, blood synovial perfusion measured by Doppler ultrasound has also been shown to be correlated with blood vessel density and disease activity (40,41), whereas antiinflammatory therapy in rat AIA is not necessarily associated with a reduction in synovial vascularization (42), and therefore might be causally unrelated.

Angiogenesis is initiated by dissolution of the perivascular surrounding matrix as well as migration and proliferation of ECs, with capillary sprouts joining to form new blood vessels. The IL-4–mediated reduction of synovial tissue blood vessels in rat AIA suggests its participation in decreased angiogenesis. To examine this possibility, we used rat AIA joint homogenates with and without IL-4–producing adenovirus for in vitro and in vivo angiogenesis assays.

First, we showed that rat AIA joint homogenates induced EC chemotaxis in vitro (Figure 2A). Interestingly, this effect was completely inhibited by AxCAIL-4, suggesting that IL-4 gene delivery results in a net angiostatic effect in vivo. Of particular importance, AxCANI, although promoting inflammation, had no impact on EC migration. Thus, it might be that the antimigratory role of IL-4 in ECs is unrelated to the antiinflammatory effects.

Second, in an in vitro capillary morphogenesis assay, we tested the role of IL-4 gene therapy on EC differentiation (Figures 2B–D). Whereas joint homogenates from the PBS- or AxCANI-treated groups induced EC tube formation in vitro, this effect was not observed with homogenates from AxCAIL-4–treated arthritic rats. This suggests that IL-4 gene delivery inhibits EC differentiation, thereby resulting in less blood vessel growth in vivo.

Third, to confirm previous data, we studied the role of AxCAIL-4–treated rat AIA joint homogenates on EC sprouting in the rat aortic ring assay (Figure 3), in which both EC migration and differentiation can be tested. Whereas significant sprouting occurred in response to joint homogenates of the PBS and AxCANI groups, IL-4 gene delivery resulted in significantly less sprout formation, suggesting that IL-4 gene delivery indeed promotes an antiangiogenic response.

Fourth, to determine if IL-4 can exert its angiostatic role in vivo, we tested the ability of rat AIA homogenates to induce blood vessel penetration and formation in a Matrigel plug in vivo angiogenesis assay. In vivo blood vessel growth was significantly inhibited in response to AxCAIL-4 homogenates compared with the PBS-treated group and tended to be lower compared with the AxCANI-treated group (Figure 4A). These findings were corroborated by the observation of significantly lower hemoglobin levels in the AxCAIL-4–treated group compared with PBS- or AxCANI-treated rats, underscoring the angiostatic properties of IL-4 in vivo (Figure 4B). In summary, IL-4 gene delivery was able to inhibit important steps of neovascularization, namely EC migration and differentiation, in vitro, as well as blood vessel formation in vivo. Thus, IL-4 may be directly or indirectly responsible for the reduction of blood vessels in the synovium in the AxCAIL-4–treated rats.

To elucidate the underlying mechanisms by which IL-4 mediates its angiostatic role, we determined the expression of select cytokines in the homogenates (Table 1). IL-18, a proangiogenic cytokine in arthritis (23), was significantly down-regulated in response to IL-4 gene therapy in rat AIA. Similarly, expression of the proangiogenic chemokine CXCL16 (29) was decreased by IL-4, while levels of the angiogenesis inhibitor endostatin were up-regulated in the AxCAIL-4–treated group compared with the group that received AxCANI treatment. These effects on endostatin were not present when the AxCAIL-4–treated group was compared with the PBS-treated group. Conceivably, the AxCAIL-4–mediated increase in endostatin levels was masked by the effects of the adenovirus itself, which significantly down-regulated joint endostatin expression. The data suggest that these proteins, at least in part, mediate the IL-4–associated antiangiogenic effects in vivo.

Moreover, expression of LIX/CXCL5, the rat homolog of the human proangiogenic chemokine ENA-78/CXCL5, was strongly down-regulated by AxCAIL-4. This is consistent with our previous data showing that preventative blocking of increased ENA-78, similar to the effects on protein levels in rat AIA, modifies the severity of AIA (43). Similarly, levels of CINC-1/CXCL1 were significantly lower in the AxCAIL-4–treated group. The human homolog of CINC-1, GROα/CXCL1, has been shown to promote angiogenesis (44). Of interest, expression of IL-6 and

IL-13 did not change following IL-4 gene delivery when compared with AxCANI, suggesting that the angiostatic effect of IL-4 in rat AIA is independent of those cytokines. Moreover, AxCAIL-4 up-regulated TGFβ in the in vivo setting of rat AIA. Indeed, TGFβ is known to have a dual role in angiogenesis, either promoting neovascularization or exhibiting angiostatic effects (45,46). Since progression of angiogenesis depends on the sum effects of pro- and antiangiogenic factors (8), our data suggest that IL-4 shifts the balance toward the angiostatic side. Of interest, the IL-4 effect was more evident in comparison with AxCANI than in comparison with PBS. This is consistent with previous observations showing that adenoviral vectors have a proinflammatory potential in vivo and can significantly affect expression of various cytokines and growth factors (12,15,47).

To study the functional relevance of our observations, we performed blocking studies for EC chemotaxis in response to AxCANI joint homogenates (Figure 4C). Neutralization of IL-18 and CXCL16 resulted in significantly reduced EC migration, an effect similar to that seen with IgG-treated AxCAIL-4 homogenates. Thus, it is very likely that the IL-4–induced reduction of CXCL16 and IL-18 contributes significantly to the angiostatic net effect of AxCAIL-4 homogenates. However, by blocking IL-4 with anti–IL-4 in the in vitro morphogenesis assay, we showed that this cytokine is also able to exert its angiostatic effects directly (Figure 4D), consistent with previous data (2,3). As expected, blocking of already-low levels of IL-18 and CXCL16 in the AxCAIL-4 homogenates did not affect EC tube formation. This finding emphasizes the notion that IL-4 may exert its angiostatic effects both directly and indirectly.

Surprisingly, in this study, IL-4 significantly inhibited neovascularization in the presence of high VEGF levels. Although this seems to contradict the paradigm of VEGF being a key player in angiogenesis, there are plausible explanations for this observation. 1) The IL-4–induced angiostatic response is the net balance of changes in levels of pro- and antiangiogenic mediators in rat AIA synovial tissue. In fact, endostatin has been shown to block VEGF-mediated signaling via direct interaction with the VEGF receptor, Flk-1, in ECs (48), and thus, if up-regulated, may neutralize the proangiogenic properties of VEGF. 2) IL-4 inhibits VEGF-mediated neovascularization, a notion that is supported by a recent study in which VEGF-induced EC chemo-

taxis and formation of tube-like structures by ECs in vitro was significantly down-regulated by IL-4 (3), while in the absence of VEGF, IL-4 enhanced EC tube formation, suggesting an angiogenic activity of IL-4 under certain conditions. 3) IL-4 down-regulates the expression of VEGF-specific receptors on ECs. IL-4 would thus reduce responses to VEGF, which possibly represents a mechanism for negative feedback regulation of angiogenesis, as has been demonstrated for $\alpha v\beta3$ integrin (49).

Since αv integrins are important regulators of angiogenesis, we determined whether IL-4 gene therapy could alter expression of integrins $\alpha v\beta3$ and $\alpha v\beta5$ on rat AIA synovial tissue (Figure 5). Expression of the integrin αv chain on ECs was significantly down-regulated by IL-4. In contrast, synovial expression of the integrin $\beta5$ chain was not significantly altered by IL-4 gene delivery. These observations, together with evidence that IL-4 regulates integrin $\alpha v\beta3$ promoter activities (50), suggest that IL-4 is able to exert its antiangiogenic effects in rat AIA synovium via integrin $\alpha v\beta3$, but not $\alpha v\beta5$.

In conclusion, we have shown in rat AIA that IL-4 gene delivery results in antiangiogenic effects in vitro and in vivo, the IL-4–induced antiangiogenic effects may be mediated directly or indirectly via alterations in the expression of pro- and antiangiogenic factors, and IL-4 may inhibit VEGF-mediated angiogenesis and exert its antiangiogenic activity via integrin $\alpha v\beta3$. This study shows that IL-4 gene therapy is a useful approach to the reduction of neovascularization in arthritis. Since preventative treatment with IL-4 is not feasible in humans, further studies are needed to determine how IL-4–associated angiostatic effects can be directed to target treatment of inflammatory arthritis in humans.

REFERENCES

1. Brown MA, Hural J. Functions of IL-4 and control of its expression. Crit Rev Immunol 1997;17:1–32.
2. Volpert OV, Fong T, Koch AE, Peterson JD, Waltenbaugh C, Tepper RI, et al. Inhibition of angiogenesis by interleukin 4. J Exp Med 1998;188:1039–46.
3. Lee IY, Kim J, Ko EM, Jeoung EJ, Kwon YG, Choe J. Interleukin-4 inhibits the vascular endothelial growth factor- and basic fibroblast growth factor-induced angiogenesis in vitro. Mol Cells 2002;14:115–21.
4. Saleh M, Davis ID, Wilks AF. The paracrine role of tumour-derived mIL-4 on tumour-associated endothelium. Int J Cancer 1997;72:664–72.
5. Toi M, Harris AL, Bicknell R. Interleukin-4 is a potent mitogen for capillary endothelium. Biochem Biophys Res Commun 1991;174:1287–93.

6. Fukushi J, Morisaki T, Shono T, Nishie A, Torisu H, Ono M, et al. Novel biological functions of interleukin-4: formation of tube-like structures by vascular endothelial cells in vitro and angiogenesis in vivo. Biochem Biophys Res Commun 1998;250:444–8.

7. FitzGerald O, Soden M, Yanni G, Robinson R, Bresnihan B. Morphometric analysis of blood vessels in synovial membranes obtained from clinically affected and unaffected knee joints of patients with rheumatoid arthritis. Ann Rheum Dis 1991;50:792–6.

8. Szekanecz Z, Gaspar L, Koch AE. Angiogenesis in rheumatoid arthritis. Front Biosci 2005;10:1739–53.

9. Miossec P, Naviliat M, Dupuy d'Angeac A, Sany J, Banchereau J. Low levels of interleukin-4 and high levels of transforming growth factor β in rheumatoid synovitis. Arthritis Rheum 1990;33:1180–7.

10. Firestein GS, Alvaro-Gracia JM, Maki R. Quantitative analysis of cytokine gene expression in rheumatoid arthritis. J Immunol 1990;144:3347–53.

11. Ohmura K, Nguyen LT, Locksley RM, Mathis D, Benoist C. Interleukin-4 can be a key positive regulator of inflammatory arthritis. Arthritis Rheum 2005;52:1866–75.

12. Woods JM, Katschke KJ, Volin MV, Ruth JH, Woodruff DC, Amin MA, et al. IL-4 adenoviral gene therapy reduces inflammation, proinflammatory cytokines, vascularization, and bony destruction in rat adjuvant-induced arthritis. J Immunol 2001;166:1214–22.

13. Allen JB, Wong HL, Costa GL, Bienkowski MJ, Wahl SM. Suppression of monocyte function and differential regulation of IL-1 and IL-1ra by IL-4 contribute to resolution of experimental arthritis. J Immunol 1993;151:4344–51.

14. Kageyama Y, Koide Y, Uchijima M, Nagata T, Yoshida A, Taiki A, et al. Plasmid encoding interleukin-4 in the amelioration of murine collagen-induced arthritis. Arthritis Rheum 2004;50:968–75.

15. Lubberts E, Joosten LA, van Den Bersselaar L, Helsen MM, Bakker AC, van Meurs JB, et al. Adenoviral vector-mediated overexpression of IL-4 in the knee joint of mice with collagen-induced arthritis prevents cartilage destruction. J Immunol 1999;163:4546–56.

16. Hynes RO. A reevaluation of integrins as regulators of angiogenesis. Nat Med 2002;8:918–21.

17. Storgard CM, Stupack DG, Jonczyk A, Goodman SL, Fox RI, Cheresh DA. Decreased angiogenesis and arthritic disease in rabbits treated with an αvβ3 antagonist. J Clin Invest 1999;103:47–54.

18. Kitazawa S, Ross FP, McHugh K, Teitelbaum SL. Interleukin-4 induces expression of the integrin αvβ3 via transactivation of the β3 gene. J Biol Chem 1995;270:4115–20.

19. Saito I, Oya Y, Yamamoto K, Yuasa T, Shimojo H. Construction of nondefective adenovirus type 5 bearing a 2.8-kilobase hepatitis B virus DNA near the right end of its genome. J Virol 1985;54:711–9.

20. Miyazaki J, Takaki S, Araki K, Tashiro F, Tominaga A, Takatsu K, et al. Expression vector system based on the chicken β-actin promoter directs efficient production of interleukin-5. Gene 1989;79:269–77.

21. Koch AE, Kurata H, Hosaka S, Woods JM, Halloran MM, Arai KI. Use of the rat adjuvant-induced arthritis as a model for rheumatoid arthritis gene therapy. Transgenics 1998;2:299–309.

22. Koch AE, Halloran MM, Haskell CJ, Shah MR, Polverini PJ. Angiogenesis mediated by soluble forms of E-selectin and vascular cell adhesion molecule-1. Nature 1995;376:517–9.

23. Park CC, Morel JC, Amin MA, Connors MA, Harlow LA, Koch AE. Evidence of IL-18 as a novel angiogenic mediator. J Immunol 2001;167:1644–53.

24. Diglio CA, Grammas P, Giacomelli F, Wiener J. Angiogenesis in rat aorta ring explant cultures. Lab Invest 1989;60:523–31.

25. Amin MA, Volpert OV, Woods JM, Kumar P, Harlow LA, Koch AE. Migration inhibitory factor mediates angiogenesis via mito-gen-activated protein kinase and phosphatidylinositol kinase. Circ Res 2003;93:321–9.

26. Ruth JH, Volin MV, Haines GK 3rd, Woodruff DC, Katschke KJ Jr, Woods JM, et al. Fractalkine, a novel chemokine in rheumatoid arthritis and in rat adjuvant-induced arthritis. Arthritis Rheum 2001;44:1568–81.

27. Volin MV, Szekanecz Z, Halloran MM, Woods JM, Magua J, Damergis JA Jr, et al. PECAM-1 and leukosialin (CD43) expression correlate with heightened inflammation in rat adjuvant-induced arthritis. Exp Mol Pathol 1999;66:211–9.

28. Shahrara S, Amin MA, Woods JM, Haines GK, Koch AE. Chemokine receptor expression and in vivo signaling pathways in the joints of rats with adjuvant-induced arthritis. Arthritis Rheum 2003;48:3568–83.

29. Zhuge X, Murayama T, Arai H, Yamauchi R, Tanaka M, Shimaoka T, et al. CXCL16 is a novel angiogenic factor for human umbilical vein endothelial cells. Biochem Biophys Res Commun 2005;331:1295–300.

30. McClintock JY, Wagner EM. Role of IL-6 in systemic angiogenesis of the lung. J Appl Physiol 2005;99:861–6.

31. O'Dell JR. Therapeutic strategies for rheumatoid arthritis. N Engl J Med 2004;350:2591–602.

32. Miossec P, van den Berg W. Th1/Th2 cytokine balance in arthritis. Arthritis Rheum 1997;40:2105–15.

33. te Velde AA, Huijbens RJ, Heije K, de Vries JE, Figdor CG. Interleukin-4 (IL-4) inhibits secretion of IL-1β, tumor necrosis factor α, and IL-6 by human monocytes. Blood 1990;76:1392–7.

34. Hart PH, Vitti GF, Burgess DR, Whitty GA, Piccoli DS, Hamilton JA. Potential antiinflammatory effects of interleukin 4: suppression of human monocyte tumor necrosis factor α, interleukin 1, and prostaglandin E2. Proc Natl Acad Sci U S A 1989;86:3803–7.

35. Van de Loo FA, Smeets RL, van den Berg WB. Gene therapy in animal models of rheumatoid arthritis: are we ready for the patients? Arthritis Res Ther 2004;6:183–96.

36. Afuwape AO, Feldmann M, Paleolog EM. Adenoviral delivery of soluble VEGF receptor 1 (sFlt-1) abrogates disease activity in murine collagen-induced arthritis. Gene Ther 2003;10:1950–60.

37. Yin G, Liu W, An P, Li P, Ding I, Planelles V, et al. Endostatin gene transfer inhibits joint angiogenesis and pannus formation in inflammatory arthritis. Mol Ther 2002;5:547–54.

38. Hirohata S, Sakakibara J. Angioneogenesis as a possible elusive triggering factor in rheumatoid arthritis. Lancet 1999;353:1331.

39. Walsh DA, Wade M, Mapp PI, Blake DR. Focally regulated endothelial proliferation and cell death in human synovium. Am J Pathol 1998;152:691–702.

40. Hau M, Schultz H, Tony HP, Keberle M, Jahns R, Haerten R, et al. Evaluation of pannus and vascularization of the metacarpophalangeal and proximal interphalangeal joints in rheumatoid arthritis by high-resolution ultrasound (multidimensional linear array). Arthritis Rheum 1999;42:2303–8.

41. Walther M, Harms H, Krenn V, Radke S, Faehndrich TP, Gohlke F. Correlation of power Doppler sonography with vascularity of the synovial tissue of the knee joint in patients with osteoarthritis and rheumatoid arthritis. Arthritis Rheum 2001;44:331–8.

42. Coxon A, Bolon B, Estrada J, Kaufman S, Scully S, Rattan A, et al. Inhibition of interleukin-1 but not tumor necrosis factor suppresses neovascularization in rat models of corneal angiogenesis and adjuvant arthritis. Arthritis Rheum 2002;46:2604–12.

43. Halloran MM, Woods JM, Strieter RM, Szekanecz Z, Volin MV, Hosaka S, et al. The role of an epithelial neutrophil-activating peptide-78-like protein in rat adjuvant-induced arthritis. J Immunol 1999;162:7492–500.

44. Strieter RM, Polverini PJ, Kunkel SL, Arenberg DA, Burdick MD, Kasper J, et al. The functional role of the ELR motif in CXC chemokine-mediated angiogenesis. J Biol Chem 1995;270:27348–57.

45. Hanahan D, Folkman J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell 1996;86:353–64.

46. Clavel G, Bessis N, Boissier MC. Recent data on the role for angiogenesis in rheumatoid arthritis. Joint Bone Spine 2003;70: 321–6.

47. Woods JM, Amin MA, Katschke KJ Jr, Volin MV, Ruth JH, Connors MA, et al. Interleukin-13 gene therapy reduces inflammation, vascularization, and bony destruction in rat adjuvant-induced arthritis. Hum Gene Ther 2002;13:381–93.

48. Kim YM, Hwang S, Pyun BJ, Kim TY, Lee ST, Gho YS, et al. Endostatin blocks vascular endothelial growth factor-mediated signaling via direct interaction with KDR/Flk-1. J Biol Chem 2002;277:27872–9.

49. Reynolds LE, Wyder L, Lively JC, Taverna D, Robinson SD, Huang X, et al. Enhanced pathological angiogenesis in mice lacking $\beta3$ integrin or $\beta3$ and $\beta5$ integrins. Nat Med 2002;8:27–34.

50. McHugh KP, Kitazawa S, Teitelbaum SL, Ross FP. Cloning and characterization of the murine $\beta(3)$ integrin gene promoter: identification of an interleukin-4 responsive element and regulation by STAT-6. J Cell Biochem 2001;81:320–32.