IL-4 Adenoviral Gene Therapy Reduces Inflammation, Proinflammatory Cytokines, Vascularization, and Bony Destruction in Rat Adjuvant-Induced Arthritis


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IL-4 Adenoviral Gene Therapy Reduces Inflammation, Proinflammatory Cytokines, Vascularization, and Bony Destruction in Rat Adjuvant-Induced Arthritis


IL-4 is a cytokine with anti-inflammatory properties on activated macrophages. Rheumatoid arthritis, an autoimmune inflammatory disease, is characterized by a paucity of IL-4 and an abundance of synovial macrophage-derived mediators. Herein, the effect of a single injection of adenovirus-producing rat IL-4 (AxCAIL-4) or a control virus with no inserted gene was compared with the effect of PBS injection into rat ankles. Ankles were injected before arthritis onset or at maximal inflammation. Preventatively, AxCAIL-4 reduced adjuvant-induced arthritis (AIA)- and/or AIA/adenoviral-induced ankle inflammation, decreasing articular index scores, ankle circumferences, paw volumes, radiographic scores, mean levels of monocyte chemoattractant protein-1, the number of inflammatory cells, and the number of synovial blood vessels. Therapeutically, AxCAIL-4 also decreased ankle circumferences and paw volumes in comparison with a control virus with no inserted gene and PBS groups. After arthritis onset, mean levels of TNF-α, IL-1β, macrophage inflammatory protein-2, and RANTES were decreased in AxCAIL-4 rat ankle homogenates compared with PBS-treated homogenates. Thus, increased expression of IL-4 via gene therapy administered in a preventative and/or therapeutic manner reduced joint inflammation, synovial cellularity, levels of proinflammatory cytokines, vascularization, and bony destruction in rat AIA, suggesting that a similar treatment in humans may be beneficial. The Journal of Immunology, 2001, 166: 1214–1222.

C hronic inflammation in rheumatoid arthritis (RA) joints is driven by a variety of proinflammatory cytokines, chemokines, and other mediators that outnumber or outperform their anti-inflammatory counterparts. Rat adjuvant-induced arthritis (AIA) is a commonly used model of RA, autoimmune disease, and inflammation. This model involves a single injection of CFA into an area of potent lymphatic drainage in susceptible rats that results in arthritis-like symptoms for many weeks in distal joints (1). Studies on the regulation of inflammation in AIA and its amelioration via novel therapies may result in improved therapeutic approaches for RA patients.

IL-4 is a pleiotropic cytokine that plays a number of important roles, including the regulation of inflammation (2). IL-4 production in normal tissue is tightly regulated and mainly occurs in Th2 cells, mast cells, and basophils (2). The RA synovium, however, lacks IL-4 (3), in addition to many other T cell-derived cytokines, despite the abundance of T cells in the synovial infiltrate (4). IL-4 acts on LPS-stimulated monocytes in vitro to down-regulate the production of the inflammatory cytokines TNF-α, IL-1α, IL-1β, IL-6, IL-8, G-CSF, and macrophage inflammatory protein (MIP)-1α (5). Complementary to its anti-inflammatory properties on monocytes, IL-4 inhibits RA synoviocyte proliferation and production of PGE2 and GM-CSF (6). IL-4 enhances monocyte apoptosis, thereby decreasing monocyte accumulation (7), and acts as an autocrine growth factor promoting the differentiation of naive T cells to Th2 cells (2). Interestingly, both IL-4- and IL-4/IL-13-deficient mice can still employ compensatory mechanisms enabling a Th2-like response, as demonstrated by the production of IL-5 (8). We have recently found that in a human ex vivo model of RA, using adenovirally produced human IL-4, we are able to markedly lower the secretion of proinflammatory mediators in the synovium (9).

By bypassing the initiating factors in RA and manipulating the cytokine balance may be an effective therapeutic means by which chronic inflammation can be managed (10). The outlook is favorable for the combination of RA treatment with gene therapy as a means for agent delivery, making the appropriate selection of candidate therapeutic proteins essential (11–13). Here, we use an adenoviral gene therapy approach in the rat AIA model to increase the expression of IL-4 in inflamed joints. We demonstrate that adenovirally produced IL-4 can reduce AIA-induced inflammation and the presence of inflammatory cells in the synovium, decrease bony destruction, reduce the quantity of synovial blood vessels, and attenuate proinflammatory cytokine levels in vivo.

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3 Abbreviations used in this paper: RA, rheumatoid arthritis; AxCAIL-4, adenovirus containing the IL-4 gene; AxCANI, adenovirus containing no inserted gene; AIA, adjuvant-induced arthritis; MIP, macrophage inflammatory protein; i.a., intra-articular; AI, articular index; MCP-1, monocyte chemoattractant protein-1; CIA, collagen-induced arthritis.

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Materials and Methods

Experimental setup

Two experimental designs, including preventative and therapeutic manipulations of AIA, were used to determine whether an adenovirus containing no inserted gene (AxCAIL-4) could prevent or treat AIA-associated inflammation. Rats for the preventative study were arbitrarily divided into three groups on day 0, including an AxCAIL-4 group as well as two control groups designed to receive PBS or an adenovirus containing no inserted gene (AxCANI; n = 10 rats/group). The PBS group was included to assure that anti-inflammatory IL-4 did not simply decrease inflammation relative to a mildly inflammatory adenoviral control without actual effects on AIA-induced swelling. Previous experiments with the rat AIA model demonstrated that maximal inflammation occurred around day 18 postadjuvant. Therefore, on day 8 after AIA induction, 5 x 10^5 or 1 x 10^6 PFUs of the adenoviral vector with or without the IL-4 gene or PBS was administered to each ankle in a 10-μl volume via intra-articular (i.a.) injection. Clinical parameters were assessed on days 0, 2, 4, and 7 after adjuvant injection (before i.a. injection) as well as days 9, 11, 14, 16, and 18 after adjuvant injection. All animals were sacrificed, and ankles were collected for further examination on day 18.

Rats for the therapeutic study were likewise divided into three groups on day 0, including an AxCAIL-4 group as well as PBS and AxCANI groups (n = 13 rats/group). Because we were examining the reduction of inflammation, on day 18 we selected the rats with the most inflamed ankles, based on ankle circumference and paw volume in each group, for i.a. injection, which resulted in a minimum of 10 rats/group. All animals were administered an i.a. injection (1 x 10^5 PFUs in 10 μl to each ankle) of the appropriate test group or PBS (10 μl) on day 18 to determine whether AxCAIL-4 could ameliorate maximal arthritis. Clinical parameters were assessed on days 0, 2, 4, 7, 9, 11, 14, 16, and 18 postadjuvant (before adeno/vascular control injection) as well as days 21, 23, and 25 postadjuvant. All animals were sacrificed, and ankles were collected for further examination on day 25.

Preparation, propagation, purification, and titration of adenoviruses

Replication incompetent AxCAIL-4 and AxCANI were prepared via homologous recombinant in 293 cells using methods described previously (14). In short, expression of the rat IL-4 gene was directed by the chicken β-actin promoter and the CMV enhancer of pCMXaWt, a 45-kb cosmid containing the full-length sequence of type 5 adenovirus deleted of the E1A, E1B, and E3 regions (15, 16). The production of rat IL-4 protein was examined by ELISA in conditioned medium of rat synovial fibroblast cultures. Viruses were propagated through successive infection of 293 cells followed by harvesting of cell lysates. Purification of virus was accomplished using cesium chloride density ultracentrifugation followed by dialysis. Titer was estimated by the number of PFUs of virus in 293 cells.

Induction of rat AIA

Female Lewis rats (100 g) were injected s.c. with 300 μl (5 mg/ml) of lypophilized Mycobacterium butyricum (Difco, Detroit, MI) at the base of the tail. All time points were considered relative to this AIA induction day, which was designated day 0. The time course and the expression of an adenoviral vector in the rat AIA model have previously been examined (14).

Clinical measurements

Clinical parameters were assessed on the days detailed above and included measurements of body weight, articular index (AI) scores, ankle circumference, and paw volume. AI scores were assigned to every joint by a single observer blinded to the group of the animal. Scoring was performed on a 0–4 scale where 0 = no swelling or erythema, 1 = slight swelling and/or erythema, 2 = low to moderate edema, 3 = pronounced edema with limited joint usage, and 4 = excess edema with joint rigidity. For ankle circumference determination, two perpendicular diameters of the joint were measured with a caliper (Lange Caliper, Cambridge Scientific Industries, Cambridge, MA). Ankle circumference was determined using the geometric formula: circumference = 2π(√(a^2 + b^2)/2), where a is the lateral-lateral diameter, and b is the antero-posterior diameter, as we have previously described (17). The volume of hind ankles was determined using a paw volume plethysmometer (Kent Scientific, Litchfield, CT).

Ankle x-rays and radiographic scoring

Upon sacrifice, ankles were promptly removed to ice, and x-rays were taken. Ankles were positioned over a radiographic cassette containing standard veterinary x-ray film (Animal Care Products, St. Paul, MN) to obtain a lateral view. A conventional x-ray source (Mobile 225, General Electric, Milwaukee, WI) was used at exposure factors of 40 kV (peak) and 25 mA for 0.1 s. Radiographs were scored on a scale of 0–3 based on joint space narrowing between the tibia and calcaneus, the tibia and talus, and the talus and calcaneus (0 = no increase in space, 3 = maximal increased joint space). Radiographs were also scored for the degree of bony destruction/erosions (from 0–4), assigning a point for an erosion in the tibia, calcaneus, talus, and metatarsals (considered together). For example, the maximum score an ankle could receive was 4 if an erosion was present in the tibia, calcaneus, talus, and any one or more of the metatarsals. Soft tissue swelling was also scored on a scale of 0–3 (0 = mild or no swelling, 2 = moderate swelling, 3 = severe swelling), where the maximum score a single ankle could receive was 3. All scores were calculated by an observer blinded to the experimental groups.

Ankle homogenates and ELISAs

After x-rays were taken, ankles for ELISA use were skinned, weighed, and frozen at –80°C. Ankles were homogenized in a 50-mL conical centrifuge tube containing 3 mL of Complete Mini protease inhibitor cocktail (Roche, Indianapolis, IN) homogenization buffer. Ankle homogenization was completed on ice using a motorized homogenizer, followed by 30 s of sonication. Homogenates were centrifuged at 2000 x g for 10 min, filtered through a 0.45-μm pore size Millipore filter (Bedford, MA), and stored at –80°C until use.

Ankle homogenate and ELISA levels in ankle homogenates were determined using commercially available ELISA kits that specifically recognize the rat cytokines TNF-α, IL-1β, monocyte chemoattractant protein-1 (MCP-1), MIP-2, and RANTES (BioSource International, Camarillo, CA) according to the manufacturer’s procedure. Rat IL-4 levels were determined using an ELISA designed with matched Ab pairs available from PharMingen (San Diego, CA). Briefly, 96-well plates were coated with mouse anti-rat IL-4 (2 μg/ml) overnight at 4°C in coating buffer (50 mM HEPES, 120 mM NaCl, pH 8.6) and washed with wash buffer (PBS and 0.05%/Tween-20). Wells were blocked with block buffer (PBS and 2% BSA) for 1 h at 37°C and washed. Reombinant rat IL-4 (R&D Systems, Minneapolis, MN) and ankle homogenates were added in duplicate to wells for 3 h at 37°C, followed by washing. Biotinylated rabbit anti-rat IL-4 (1 μg/ml) in block buffer was incubated for 1 h at 37°C and washed, followed by incubation with streptavidin-HRP (PharMingen; 1/100,000) in block buffer. After 30 min wells were washed, and HRP was detected with 3,3′,5,5′-tetramethyl-benzidine liquid substrate system (Sigma, St. Louis, MO). Color development was terminated with 0.5 M H2SO4, and plates were read at 450 nm.

Histologic analysis of tissue sections

After x-rays were taken, ankles for sectioning were stripped, mounted in OCT (Miles, Elkhart, IN), and frozen at –80°C until sectioning. Sections (3 μm) were cut using a knife suitable for bone cutting and stained with hematoxylin and eosin. The synovial infiltrate, including monocyte/macrophages, lymphocytes, and polymorphonuclear cells, was determined, based on characteristic morphologic features, by a pathologist, blinded to the experimental groups. The sum of cell counts as well as the number of blood vessels in three ×1000 microscopic fields were determined for each section.

Statistical analysis

Rats treated in the preventative and therapeutic manner described above received identical injections in each ankle based on their group assignment (PBS, AxCANI, or AxCAIL-4). As in previous studies, AIA rats often developed inflammation to different degrees in each of the hind limbs, as demonstrated by different AI scores. For this reason, each ankle was treated independently for statistical purposes. By Student’s t test, p < 0.05 was considered significant.

Results

Dose-response studies with AxCANI and AxCAIL-4

Several concentrations of AxCANI, namely 5 x 10^5, 1 x 10^7, 5 x 10^7, and 1 x 10^8 PFU, were injected i.a. into AIA as well as adjuvant-naive rats to assess induction of ankle inflammation. Adjuvant-naive rats were injected with AxCANI at various doses on day 0, and their weights, AI scores, and ankle circumferences were measured over this period received a zero, indicating that no dose of AxCANI induced inflammation (data not shown). Similarly, ankle circumferences measured over this period
did not vary significantly among the groups (Fig. 1A). Further, rat body weight was not significantly altered among the groups (data not shown). In contrast, when AIA rats were injected with the same doses of virus 8 days postadjuvant injection, but before signs of swelling, AxCANI clearly induced ankle inflammation when examining ankle circumference (Fig. 1B) and AI scores (Fig. 1C). It appears that a cut-off point exists between the lowest dose used (5 × 10^6 PFU), which does not appear to induce inflammation, and the 1 × 10^7 PFU dose, which does.

Assessment of preventative AxCAIL-4 treatment at 1 × 10^8 PFU

Adenovirus was initially investigated at 1 × 10^6 PFUs because this quantity of IL-4-producing virus has previously been shown to significantly reduce the production of proinflammatory mediators in an ex vivo model of RA (9) and because this dose was used effectively in previous rat models of arthritis (18, 19). Rat ankle homogenates were analyzed for IL-4 production by ELISA on day 18, 10 days after i.a. injections of virus. The mean IL-4 levels of preventative treated AxCAIL-4 ankles (5.3 ± 1.1 ng/ml; n = 9) were 6.6- and 8.8-fold higher than the mean levels in PBS (0.8 ± 0.05 ng/ml; n = 6) and AxCANI (0.6 ± 0.05 ng/ml; n = 5) groups, respectively. Animal body weights were determined three times per week in the 18-day study (Fig. 2). Mean body weights of rats in all three groups increased through day 7, before i.a. injections. After day 8 the mean body weights of PBS-injected rats remained consistent for about 6 days and then decreased. Weight loss before detection of clinical signs of inflammation in the hind limbs is one of the systemic AIA effects and was an anticipated result. In contrast, animals that did not receive adjuvant continued to increase in body weight throughout this period following a sham injection (20). Mean body weights of AxCANI rats declined immediately after adenoviral administration. In contrast, mean body weights of AxCAIL-4 rats remained approximately constant for 3 days postinjection, and then increased and remained consistent for the duration of the study. By comparison with the AxCANI group, mean AxCAIL-4 rat weights were significantly higher just 1 day after adenoviral administration and remained significantly higher for the duration of the study (p < 0.05). Compared with weights in the control group that received AIA and a sham PBS i.a. injection, mean AxCAIL-4 rat weights were significantly higher from days 14 through 18, perhaps indicative of healthier animals.

In addition to body weights, AI scores, ankle circumferences, and paw volume were determined regularly throughout the study. AI scores were rated on a scale of 0–4 for each ankle injected. The mean AI scores for PBS-injected ankles (Fig. 3A) show that AIA-induced swelling began around day 10 and increased through day 18. By comparison, 1 × 10^8 PFU of adenovirus induced an inflammatory response immediately after injection, as mean AI scores were higher than that of the PBS-injected group (p < 0.05). After day 9, however, mean AI scores for the AxCANI and AxCAIL-4 groups were higher than that in the PBS-injected group (p < 0.05). By comparison with the AxCANI group, mean AI scores for the AxCAIL-4 groups quickly diverged as scores for ankles receiving IL-4 leveled off for the remainder of the study. From days 11 through 18, the mean AI score of AxCAIL-4-injected animals was significantly lower than that of the AxCANI control (p < 0.05). Further, on day 16 the mean AI score of AxCAIL-4 rats was significantly below that of the PBS group.

FIGURE 1. AxCANI, at doses of 5 × 10^6, 1 × 10^7, 5 × 10^7, and 1 × 10^8 PFU, were injected i.a. into AIA as well as adjuvant-naive rats to assess induction of ankle inflammation. Values represent the mean ± SE of the indicated measurement. A. Ankle circumferences of adjuvant-naive rats who were injected i.a. with AxCANI on day 0 were measured at nine different time points. B. Ankle circumferences of AIA rats injected i.a. with AxCANI 8 days postadjuvant injection were measured on 10 different days. C. AI scores were assigned to each rat hind limb on 10 different days over the 26-day time course. *, Difference between the 1 × 10^6 and 5 × 10^6 PFU doses (p < 0.05); **, difference between the 5 × 10^6 and 5 × 10^7 PFU doses (p < 0.05).

FIGURE 2. Rats receiving preventative administration of AxCAIL-4 had higher body weights than those receiving PBS or AxCANI. Rats were administered adjuvant on day 0 and were injected i.a. with AxCAIL-4, AxCANI, or PBS on day 8 (arrow). Body weights of rats were determined three times per week throughout the 18-day study. Values represent the mean ± SE rat body weight. *, Difference between the AxCANI and AxCAIL-4 groups (p < 0.05); **, difference between the PBS and AxCAIL-4 groups (p < 0.05).
Ankle circumference is another accurate way to monitor rat ankle inflammation. Before adenoviral injections, mean ankle circumferences were similar among groups (Fig. 3B). On day 9, 1 day after ankle injections, the mean ankle circumferences for the AxCAIL-4 group were significantly higher than those for the PBS group \((p < 0.05)\). By day 11, however, mean ankle circumferences for PBS and AxCAIL-4 animals were identical. Beyond day 11, the mean ankle circumference of AxCAIL-4 rats was lower than that of the PBS group, attaining statistical significance on day 16 \((p < 0.05)\). Comparing the mean ankle circumference of AxCAIL-4 rats with that of the AxCANI group demonstrates that IL-4-treated rats had significantly smaller ankles from days 9 through 18 \((p < 0.05)\).

Plethysmometer measurements of paw volume were also used to compare the relative quantity of inflammation among groups. This measurement was used because it provides an objective physical measurement of total paw swelling, whereas ankle circumference does not take swelling in the lower foot into account, and the AI scores are a subjective measurement. Mean paw volume measurements on days 11 and 16 demonstrated that AxCAIL-4 conferred an anti-inflammatory effect less than that in the PBS-injected group (Fig. 3C). Further, mean paw volumes of the AxCAIL-4 group were significantly less than those in the AxCANI group from days 14 through 18. These results and the consistency of the three methods for measuring rat paw inflammation strongly suggest that IL-4 is capable of reducing clinical symptoms of rat AIA.

An examination of rat ankle x-rays for bony erosion/destruction and soft tissue swelling was also performed on day 18. When x-rays from the preventatively treated group were graded for bone erosions alone, the mean score for the AxCAIL-4–treated group \((0 \pm 0)\) was significantly lower than that for the PBS \((0.8 \pm 0.3)\) or AxCANI \((2.1 \pm 0.3)\) group \((p < 0.05)\). As indicated by the mean, no AxCAIL-4 preventatively treated ankles were found to contain bone erosions. When scored for joint space narrowing alone, the mean score for the AxCAIL-4–injected group \((0.3 \pm 0.1)\) was also lower than that for the PBS-injected \((1.1 \pm 0.2)\) or AxCANI-injected \((1.9 \pm 0.2)\) group \((p < 0.05)\). When scored for soft tissue swelling alone, the mean AxCAIL-4 score \((1.5 \pm 0.1)\) was significantly lower than that of the AxCANI-injected group \((2.5 \pm 0.2, p < 0.05)\), but not that of the PBS group \((1.5 \pm 0.2)\). Fig. 4 depicts the sum of all the x-ray scores (bony erosion, joint space narrowing, and soft tissue swelling), showing mean AxCAIL-4 scores 48 and 73% lower than those of PBS and AxCANI controls, respectively \((p < 0.05)\). The data suggest that the production of IL-4 in the AIA joint can prevent both AIA- and AIA/adenovirus-induced destruction of bone.

Prolinflammatory cytokines probably play a role in persistent inflammation and therefore were examined in rat ankle homogenates by ELISA. Cytokines were normalized to total protein (Table I), while normalization to prehomogenized ankle weight yielded similar results (data not shown). There were obvious differences in ankle sizes among the various treatment groups, which was reflected in ankle weights. Mean ankle weights \((\pm SE)\) of preventatively treated PBS, AxCANI, and AxCAIL-4 rats were \(1.0 \pm 0.2, 1.3 \pm 0.1,\) and \(0.8 \pm 0.1\) g, respectively. The mean total protein concentrations \((\pm SE)\) of preventatively treated PBS, AxCANI, and AxCAIL-4 rat ankles were \(5.4 \pm 0.8, 8.2 \pm 0.4,\) and \(4.0 \pm 0.2\) mg/ml, respectively. It is noteworthy that the protein concentration of AxCAIL-4 ankles was significantly higher than those in the PBS group.

**FIGURE 3.** Preventatively administered AxCAIL-4 reduced AIA- and AIA/adenovirus-induced inflammation. Rats were administered adjuvant on day 0 and were injected i.a. with AxCAIL-4, AxCANI, or PBS on day 8 (arrow). Values represent the mean \(\pm SE\) of the indicated measurement. *Difference between the AxCANI and AxCAIL-4 groups \((p < 0.05)\); **difference between the PBS and AxCAIL-4 groups \((p < 0.05)\). A, AI scores were assigned to each rat hind limb on 9 different days over the 18-day time course. B, Ankle circumferences were determined from perpendicular caliper measurements of ankle diameter using a geometric formula. C, Paw volume measurements were recorded using a plethysmometer.

**FIGURE 4.** Rats administered preventative AxCAIL-4 have lower radiographic scores than rats given PBS or AxCANI. Rat ankles were x-rayed on day 18 postadjuvant injection. Ankles were scored on a scale of 0–4 for bony erosions, 0–3 for joint space narrowing, and 0–3 for soft tissue swelling, as described in Materials and Methods. The sum of all three scores was calculated, and values represent the mean \(\pm SE\) of that sum. *Significantly different values \((p < 0.05)\).
and AxCAIL-4 groups \( (p < 0.05) \). There was no significant difference between the protein concentration of PBS- and AxCAIL-4-treated rat ankles, and consequently, cytokine data for the AxCANI group were not included in Table I, because reliable normalization was not possible. Mean MCP-1 levels were decreased by 44% in AxCAIL-4 rat ankle homogenates compared with levels in PBS-injected ankle homogenates \( (p < 0.05) \). Quantitation of TNF-\( \alpha \), IL-1\( \beta \), MIP-2, and RANTES was performed in a similar manner. Mean levels of these cytokines did not differ when normalized to protein concentration between AxCAIL-4- and PBS-injected ankles (Table I).

Histologic assessment of tissue sections from rat ankles injected preventatively was also analyzed (Fig. 5). AxCAIL-4 reduced the mean number of monocytes by 27 and 19% compared with counts from the PBS-treated \( (p < 0.05) \) and AxCANI-treated groups, respectively. Mean lymphocyte counts were likewise affected by AxCAIL-4, being reduced by 41 and 30% compared with mean counts for PBS-injected \( (p < 0.05) \) and AxCANI-injected joints, respectively. The largest decrease in cell counts was seen in mean polymorphonuclear cell numbers, where AxCAIL-4 decreased PBS-injected \( (p < 0.05) \) and AxCANI-injected counts by 64 and 48%, respectively. Finally, AxCAIL-4 reduced the mean number of blood vessels by 28 and 36% compared with mean vessel numbers from PBS and AxCANI \( (p < 0.05) \) sections.

Assessment of therapeutic AxCAIL-4 at \( 1 \times 10^8 \) PFU

Rat ankle homogenates were analyzed for IL-4 production by ELISA on day 25 postadjuvant injection, 7 days after i.a. injection. Mean IL-4 levels of therapeutically injected AxCAIL-4 ankles \( (2.4 \pm 0.4 \) ng/ml; \( n = 12 \) ) were 3.4- and 4.0-fold higher than those of PBS-treated \( (0.7 \pm 0.05 \) ng/ml; \( n = 6 \) ) and AxCANI-treated \( (0.6 \pm 0.06 \) ng/ml; \( n = 6 \) ) groups, respectively.

Rat weights were determined three times per week for the duration of the 25-day study. By day 18, the day of i.a. injection, there was a discrepancy among groups with respect to mean rat weights despite random assignment of animals to experimental groups. The PBS-injected group of rats had higher mean weights than rats of the AxCAIL-4 and AxCANI groups. Therefore, all therapeutic data are presented as a percentage of the respective group’s day 18 values. On days 23 and 25 AxCAIL-4 rats amassed significantly more weight than PBS or AxCANI rats, when determined relative to day 18 values (Fig. 6; \( p < 0.05 \)).

AI scores were also determined throughout the length of the study. Three days after therapeutic administration (day 21) the mean AI score of AxCAIL-4 rats (relative to day 18 values) was 25% \( (p < 0.05) \) and 9% lower than those of the PBS and AxCANI groups, respectively (data not shown). On day 23 the mean AI score of AxCAIL-4 rats, relative to day 18 values, was 17% and 11% lower than those of the PBS and AxCANI groups, respectively. However, neither these values nor those on day 25 (data not shown) were significantly different.

Ankle circumferences were significantly decreased at all time points measured after therapy was initiated (Fig. 7A). Mean ankle circumference values of AxCAIL-4 rats, relative to day 18 values, were 9, 12, and 6% below similar values for the PBS group on days 21, 23, and 25, respectively \( (p < 0.05 \) at all time points). Similarly, mean ankle circumference values of AxCAIL-4 rats, relative to day 18 values, were 10, 14, and 10% lower than values of PBS-treated and AxCANI-injected joints.

### Table I. Rat TNF-\( \alpha \), IL-1\( \beta \), MCP-1, MIP-2, and RANTES levels in AIA ankle homogenates from preventative and therapeutic AxCAIL-4- and PBS-treated groups

<table>
<thead>
<tr>
<th></th>
<th>Preventative Group</th>
<th>Therapeutic Group</th>
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<tbody>
<tr>
<td></td>
<td>PBS ((n = 10))</td>
<td>AxCAIL-4 ((n = 10))</td>
</tr>
<tr>
<td>TNF-( \alpha ) (pg)/protein (mg)</td>
<td>57.0 ± 23.7</td>
<td>55.0 ± 12.3</td>
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<td>IL-1( \beta ) (pg)/protein (mg)</td>
<td>18.1 ± 6.8</td>
<td>16.9 ± 2.4</td>
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<tr>
<td>MCP-1 (pg)/protein (mg)</td>
<td>19.9 ± 3.5</td>
<td>11.2 ± 1.5*</td>
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<tr>
<td>MIP-2 (pg)/protein (mg)</td>
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<td>8.6 ± 1.8</td>
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<tr>
<td>RANTES (pg)/protein (mg)</td>
<td>37.6 ± 15.8</td>
<td>30.6 ± 5.5</td>
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*Values represent the mean ± SE of the indicated cytokine normalized to protein concentration.

\* \( p < 0.05 \) between AxCAIL-4- and PBS-injected groups, within the respective treatment group.
for the AxCANI group on days 21, 23, and 25, respectively \((p < 0.05\) at all time points).

Paw volumes of therapeutically injected animals were also determined throughout the duration of the experiment. On day 21 mean paw volumes of AxCAIL-4 rats, relative to day 18 values, were 16% lower than similar values in the PBS-injected group \((p < 0.05\); data not shown). By day 23, mean paw volumes of AxCAIL-4 rats, relative to day 18 values, were 21% lower than similar values in the PBS group and 13% lower than values in the AxCANI group \((p < 0.05\); Fig. 7B).

An analysis of bony erosions for therapeutically treated rats was also performed. The mean bony erosion score of AxCAIL-4-treated ankles \((0.6 \pm 0.2)\) was significantly lower than that of AxCANI-treated ankles \((1.5 \pm 0.2; p < 0.05)\), but not that of PBS-treated ankles \((1.0 \pm 0.3)\). When scored for joint space narrowing, the mean AxCAIL-4 score \((0.9 \pm 0.2)\) was again significantly lower than that of AxCANI-treated ankles \((1.6 \pm 0.2; p < 0.05)\), but not that of PBS-treated ankles \((1.0 \pm 0.2)\). Mean soft tissue swelling scores of AxCAIL-4-treated ankles were not significantly different from those of the PBS or AxCANI groups. Fig. 8 depicts the sum of the all x-ray scores (bone erosion, joint space, and soft tissue swelling), showing mean AxCAIL-4 scores 40% lower than those of the AxCANI control \((p < 0.05)\) and only 5% lower than those of the PBS control.

Cytokine concentrations in rat ankle homogenates of therapeutically injected rats were also determined. In accordance with results obtained for preventatively injected ankles, ankles injected after arthritis onset displayed obvious differences in appearance, which were reflected in ankle weight. Mean ankle weights \((\pm \text{SE})\) of the therapy group injected with PBS, AxCANI, and AxCAIL-4 were \(0.8 \pm 0.1, 1.2 \pm 0.1, \text{and} 1.0 \pm 0.1 \text{g}\), respectively. Mean total protein concentrations \((\pm \text{SE})\) of PBS-, AxCANI-, and AxCAIL-4-treated rat ankles were \(5.2 \pm 1.0, 8.7 \pm 0.4, \text{and} 7.1 \pm 0.4 \text{mg/ml}\), respectively. Similar to preventatively injected ankles, the protein concentration of ankles treated after arthritis onset with AxCANI were significantly higher than those in the PBS and AxCAIL-4 groups \((p < 0.05)\); there were no differences between PBS- and AxCAIL-4-treated ankles. Consequently, cytokine data for the AxCANI group were not included in Table 1, because reliable normalization was not possible. Mean AxCAIL-4-treated rat ankle homogenate levels of TNF-\(\alpha\), IL-1\(\beta\), MIP-2, and RANTES (normalized to protein content) were decreased by 73, 76, 66, and 71%, respectively, compared with those in PBS-treated ankles \((p < 0.05)\). In contrast to preventatively treated ankles, AxCAIL-4 had no effect on mean MCP-1 levels when given after the development of clinical symptoms.

Histologic assessment of tissue sections from therapeutically injected rat ankles were also examined. Although AxCAIL-4 reduced the number of lymphocytes by 19% compared with that in the PBS group, therapeutic injections did not yield statistically significant reductions in any of the cell types or the number of blood vessels (data not shown).

**AxCAIL-4 at \(5 \times 10^6\) PFU**

Because the data in Fig. 1 demonstrated that \(5 \times 10^6\) PFU AxCANI did not induce an inflammatory response in AIA rats, we also examined the effect of this dose of AxCAIL-4. In contrast to our data for \(1 \times 10^8\) PFU, Fig. 9 demonstrates that the lower dose of AxCAIL-4 induces inflammation when administered before arthritis onset. The AI scores (Fig. 9A) and ankle circumferences (Fig. 9B) showed a similar trend. In contrast, the same dose given therapeutically did not have any effect on ankle circumference (Fig. 9C) or AI scores (data not shown).

**Discussion**

A recent investigation of preventative AxCAIL-4 in mouse collagen-induced arthritis (CIA) focused on synovial and cartilage destruction (21). In this model IL-4 prevented chondrocyte death and
AxCAIL-4 significantly reduced ankle homogenate levels of the circumference and paw volume. In addition, the latter treatment with ankle inflammation, as demonstrated by reductions in ankle cir-
PFU likewise inhibited AIA-associated weight loss and reduced bone integrity than AIA- or AIA/adenovirus-treated ankles, as sug-
study ankles receiving virally produced IL-4 maintained better levels of the proinflammatory cytokine MCP-1. In addition, in our
PFU decreased ankle inflammation, increased rat body weight, re-
cartilage. Despite suppressed levels of IL-1β and TNF-α, enhanced onset and aggravated synovial inflammation were found (21). In contrast, retrovirally produced IL-4 was effective when administered before arthritis onset in reducing paw swelling (22). Similar to the previous study, the latter data supported a reduction in bone destruction (22). Herein, we investigated both preventative and therapeutic approaches with AxCAIL-4 and focused on inflammatory parameters of AIA. Preventative AxCAIL-4 at 1 x 10^6 PFU decreased ankle inflammation, increased rat body weight, reduced synovial cellularity and blood vessel numbers, and lowered levels of the proinflammatory cytokine MCP-1. In addition, in our study ankles receiving virally produced IL-4 maintained better bone integrity than AIA- or AIA/adenovirus-treated ankles, as suggested by radiographic data. Therapeutic AxCAIL-4 at 1 x 10^6 PFU likewise inhibited AIA-associated weight loss and reduced ankle inflammation, as demonstrated by reductions in ankle circumference and paw volume. In addition, the latter treatment with AxCAIL-4 significantly reduced ankle homogenate levels of the proinflammatory cytokines IL-1β, TNF-α, MIP-2, and RANTES.

The actions of endogenous and exogenous IL-4 on inflammation in vivo are not clear. DBA/1J mice do not develop arthritis when induced with Mycobacterium tuberculosis over a 120-day period (23). However, administration of a neutralizing IL-4 Ab once daily for 10 days i.p. initiated at the time of M. tuberculosis immunization induced arthritis in 83% of mice by day 28 (23). Similar results occur when IL-4 is neutralized in T cell-mediated arthritis (24). These studies imply that endogenous IL-4 may inhibit arthritis development, but do not demonstrate directly that IL-4 can achieve this effect. Similarly, in CIA, injections of anti-IL-4 i.p. for 10 days initiated on the day of immunization with type II articular cartilage collagen markedly augmented the incidence and severity of arthritis (25). However, anti-IL-4 Ab did not affect CIA incidence or macroscopic arthritis evaluation in a similar study (26). Together, the previous studies suggest that a possible explanation for the preventative characteristics of IL-4 may be its immuno-
regulatory ability to decrease a Th1 and/or increase a Th2 cell profile. It is noteworthy that RA is also a Th1-dominant disease in which IL-4 levels are limited and IFN-γ secretion may be increased (3, 27–29).

In the present study preventative AxCANI induced inflammation above that produced by AIA alone, as represented by the PBS group (Fig. 3). Fig. 3 also demonstrates that production of IL-4 by adenovirus eliminated adenovirally induced inflammation, as the swelling of AxCAIL-4 ankles was less than that in the sham-injected PBS control. This suggests that inclusion of the IL-4 gene in inflammation-inducing viruses may counteract the immune response induced by the virus and its related proteins. The retention of bone integrity by AxCAIL-4-injected rats before disease onset was an additional benefit conferred by IL-4. It is possible that IL-4 reduced bony destruction by inhibiting bone resorption through its actions on osteoclasts and proinflammatory cytokines, as it does ex vivo (30). This result is in agreement with a recent paper that demonstrated that local IL-4 production by virus injection prevented joint damage and bone erosion in mice with CIA (31).

Ample evidence supports a role for IL-4 in modulating established autoimmune diseases. These include studies in nonobese diabetic mice (32), experimental allergic encephalomyelitis (33), CIA (34–36), and streptococcal cell wall arthritis (37). Herein, we demonstrate that a single AxCAIL-4 injection administered near the peak of inflammation can significantly reduce inflammation and proinflammatory cytokine production associated with rat AIA. This approach may confer some advantage over administration of cytokine via a pump. Fig. 6 demonstrates that after disease onset AxCAIL-4 significantly increased body weights 5 and 7 days after i.a. injection compared with control weights. Animals receiving AxCANI before disease onset lost more weight than PBS controls (Fig. 2), whereas the same dose of adenovirus did not have this effect after inflammation was established (Fig. 6). Similarly, Fig. 7, A and B, demonstrates that the dichotomy between the AxCANI and PBS groups was not as disparate as that with preventative administration (Fig. 3). This suggests that adenovirus does not in-
duce much additional inflammation beyond that of AIA, once the disease is at its peak. Measurements of AxCAIL-4-injected ankles (Fig. 7) clearly demonstrate that IL-4 production can significantly improve CIA- and AIA/adenovirus-induced inflammation. X-ray analysis of animals treated after arthritis onset (Fig. 8) dem-
strated that IL-4 could significantly improve AIA/adenovirus-in-
duced radiographic scores. However, IL-4 could not statistically improve scores compared with AIA-induced damage alone. It should be noted that the day 25 analysis of bones in the therapy model was performed only 7 days after the i.a. injection. Preventatively, x-ray analysis was performed on day 18, which was 10

**FIGURE 9.** AxCAIL-4 at 5 x 10^6 PFU induced inflammation when administered prophylactically, but had no effect when given therapeutically. Rats were administered adjuvant on day 0 and were injected i.a. with AxCAIL-4, AxCANI, or PBS on day 8 (A and B) or day 22 (C). Values represent the mean ± SE of the AI scores (A) or ankle circumference (B and C). *, Difference between the AxCANI and AxCAIL-4 groups (p < 0.05); **, difference between the PBS and AxCAIL-4 groups (p < 0.05).
days after the i.a. injection on day 8. It is possible that the prolonged IL-4 ankle injection of our preventatively designed experiment may account for the statistical improvement demonstrated when treatment was administered before arthritis onset.

In the therapeutic setting the reduction of inflammation may be attributed to the ability of IL-4 to abrogate production of proinflammatory cytokines. We recently investigated whether an adenovirus producing human IL-4 could reduce the production of proinflammatory mediators from RA synovial tissue explants ex vivo (9). Virally produced IL-4 significantly reduced the secretion of IL-1β, TNF-α, IL-8, MCP-1, epithelial neutrophil activating peptide-78, growth-related gene product-α, and PGE2, all of which are present in the RA synovium (9). In the present study there were some differences between the effects of IL-4 on proinflammatory cytokines in ankles when administered before and after disease onset. Preventative administration of AxCAIL-4 significantly decreased MCP-1 levels. Treatment after arthritis onset, in contrast, decreased TNF-α, IL-1β, MIP-2, and RANTES levels, but not those of MCP-1. It is possible that preventative IL-4 effects were partially achieved by inhibiting an early and essential monocyte-dependent stage of rat AIA that is MCP-1 reliant. Once the inflammatory response is established, however, IL-4 selectively inhibited a broader array of inflammatory mediators. An additional possibility for differences between the outcomes when treating before or after disease onset may be related to the differences in the amount of IL-4 produced. Comparing ankle homogenate concentrations of IL-4 in the two studies demonstrates that preventatively injected ankles contain 2.2-fold higher mean concentrations of IL-4 than ankles injected after disease onset. This is probably the result of the disparity in the paw volume that is being injected between days 8 and 18. For example, inflamed joints on day 18 (therapeutic study) had 2.0-fold greater paw volume than ankles injected on day 8 (preventative study). Therefore, optimizing the quantity of AxCAIL-4 used in therapeutic injections to produce an equivalent concentration of rat IL-4 as in preventively injected ankles may have resulted in additional significant changes, including a decrease in synovial cellularity. Despite this, the concentration of IL-4 produced was sufficient to significantly improve body weight, reduce ankle circumference, lower paw volume, and decrease quantities of proinflammatory cytokines.

A possible mechanism that could account for the actions of IL-4 may be via inhibiting neovascularization into synovial tissue. IL-4 has a biphasic dose-response curve with regard to endothelial cell migration in vitro and neovascularization in vivo (38). For example, we have shown previously that low doses of IL-4 are proangiogenic, while higher doses can inhibit neovascularization induced by basic fibroblast growth factor (38, 39). When 1 × 10⁶ PFU AxCAIL-4 was administered before arthritis onset, IL-4 production reduced the mean number of blood vessels in synovial sections by 39 and 36% vs those in PBS and AxCANi tissue sections, respectively. This could in part contribute to the decreased inflammation and cellularity of IL-4-treated synovium. When 5 × 10⁶ PFU AxCAIL-4 were administered before arthritis onset, IL-4 induced inflammation. This is in agreement with recently published results showing that 1 × 10⁶ PFU of virally produced IL-4 increased clinical and histological signs of inflammation in CIA in mice when administered preventatively, but a dose of 1 × 10⁵ PFU did not (21, 31). In short, these results underscore the critical nature of proper dosing with IL-4.

While this paper was under review a study was published that investigated virally produced IL-4 in murine CIA in a therapeutic manner (40). Local injections of 5 × 10⁶ viral particles producing murine IL-4 were shown to partially reverse the progression of established disease (40). Our results suggest that low doses (5 × 10⁶ PFU) of virally produced IL-4 in rat AIA are proinflammatory, whereas higher doses (1 × 10⁹ PFU) are anti-inflammatory when administered before arthritis onset. We show that joint vascularity as well as cytokine levels of TNF-α, IL-1β, MCP-1, RANTES, and MIP-2 are all modulated by IL-4. We demonstrate that adenovirally produced IL-4 administered before or after AIA induction can ameliorate arthritis. In comparison with animals given AIA or adenoviral/AIA treatment, animals injected with virally produced IL-4 had higher body weights, reduced inflammation, decreased synovial cellularity, and reduced bony destruction. Optimizing the proper vector, dose, and route of administration of IL-4 may result in an efficient therapeutic modality for the treatment of RA patients.

References


