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ROLE OF INTERLEUKIN-4 AND INTERLEUKIN-10 IN MURINE COLLAGEN-INDUCED ARTHRITIS

Protective Effect of Interleukin-4 and Interleukin-10 Treatment on Cartilage Destruction

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Objective. **To examine the role of endogenous interleukin-4 (IL-4) and interleukin-10 (IL-10) and the therapeutic effect of the addition of IL-4 and IL-10 in early and established murine collagen-induced arthritis (CIA).**

Methods. **Murine recombinant IL-4, IL-10, or the combination was given intraperitoneally twice daily from the day of arthritis onset up to 7–10 days of CIA in DBA/1 mice. Anti–IL-4, anti–IL-10, or both antibodies were given intraperitoneally before or after the onset of CIA. The effect of cytokine or anticytokine treatment was monitored visually by macroscopic scoring. Histology and reverse transcription–polymerase chain reaction (RT-PCR) analyses were performed at the end of the treatment period.**

Results. **IL-4 alone did not provoke any effect, IL-10 slightly suppressed the arthritis, but a more pronounced amelioration was found with the combination. This cooperative effect was noted after early treatment but also occurred when the start of treatment was delayed until 1 week after onset. Apart from suppression of macroscopic signs of inflammation, combined treatment with IL-4/IL-10 also reduced cellular infiltrates in the synovial tissue and caused pronounced protection against cartilage destruction. Moreover, levels of mRNA for tumor necrosis factor α (TNFα) and IL-1 were highly suppressed both in the synovial tissue and in the articular cartilage. In contrast, levels of IL-1 receptor antagonist (IL-1Ra) mRNA remained elevated, which suggests that the mechanism of protection may be related to suppressed production of TNFα and IL-1, with concomitant up-regulation of the IL-1Ra/IL-1 balance. However, accelerated onset of CIA and increased severity could be achieved with neutralizing anti–IL-10 antibodies. This expression could be further optimized with a combination of anti–IL-4 and anti–IL-10 antibodies, although anti–IL-4 alone was without effect.**

Conclusion. **Our data are consistent with a dominant role of IL-10 in the natural suppression of arthritis expression, whereas combined treatment with IL-4 and IL-10 appears of potential therapeutic value, not only at the onset, but also in established arthritis.**

Interleukin-4 (IL-4) and interleukin-10 (IL-10) are pleiotropic cytokines that can exert either suppressive or stimulatory effects on different cell types of the immune system. IL-4 and IL-10 were first identified as products of T helper 2 (Th2) clones and are part of the cytokines that distinguished Th2 cells from other T cells. In addition, IL-4 and IL-10 inhibited IL-2 and interferon-γ production by Th1 cells, resulting in suppression of macrophage activation (1,2). It has recently been demonstrated that IL-4 and IL-10 inhibit the production of proinflammatory cytokines, such as IL-1, IL-6, IL-8, and tumor necrosis factor α (TNFα) by monocytes and macrophages (3–6). IL-4 and IL-10 suppress cytokine synthesis by different mechanisms. IL-4 enhances messenger RNA (mRNA) degradation, without a decrease in cytokine gene transcription, while IL-10 inhibits nuclear factor κB, resulting in suppression of gene transcription (7). Furthermore, it has been shown that IL-4 and IL-10 stimulate the production of cytokine inhibitors such as IL-1 receptor antagonist

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(IL-1Ra), soluble IL-1R type II, and soluble tumor necrosis factor receptor by monocyte/macrophages and neutrophils (8–11).

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by cartilage damage and bone destruction. There is increasing evidence that TNFα and, in particular, IL-1 are involved in cartilage damage (12). These proinflammatory cytokines are detected in synovial fluid of RA patients, and the production of TNFα and IL-1 by RA synovial tissue has been demonstrated (13). Recent clinical trials with neutralizing antibodies against TNFα have demonstrated efficacy in human RA (14,15).

Collagen-induced arthritis (CIA) is a widely used experimental model of polyarthritis. It can be induced in susceptible strains of mice and rats by immunization with type II collagen, the major component of articular cartilage, and has histopathologic features in common with RA (16–18). In this animal model of arthritis, it is clearly shown that TNFα and IL-1 are involved in onset of the disease (19–20). Furthermore, neutralization of IL-1 with antibodies against IL-1 or with IL-1Ra during established CIA was shown to completely suppress the arthritis and prevent joint pathology (21–23).

Recent studies have shown that there are elevated levels of IL-10 in the synovial fluid of RA patients and that the production of IL-1 and TNFα by RA synovial tissue can be regulated by IL-4 and IL-10 (24–26). In general, attempts to detect IL-4 in RA synovial tissue suggested a relative absence of this modulator at that site. This may provide a reason for uncontrolled cytokine production in RA, and may also offer a therapeutic approach. Efficacy of the in vivo administration of IL-4 or IL-10 has already been shown in TNF/IL-1–dependent animal models, such as endotoxin-induced lethality and IgG immune complex–induced lung injury (27,28), but data in experimental arthritis are scant. Allen et al (29) showed that sustained treatment with IL-4 suppressed the chronic, but not the acute, phase of streptococcal cell wall (SCW)–induced arthritis in rats and demonstrated up-regulated IL-1Ra mRNA levels in the monocytes of these animals. Kasama et al (30) showed elevated levels of IL-10 at the onset of CIA, and a regulatory role was demonstrated by increased disease activity after neutralization of IL-10. Finally, efficacy of IL-10 treatment on the progression of murine CIA was recently reported by Walmsley et al (31).

In the present study, we investigated the effects of IL-4 and IL-10, either alone or in combination, on the treatment of early and established murine CIA. We also used neutralizing antibodies in vivo to examine the role of endogenous IL-4/IL-10 during both the onset and the established phase of this experimental arthritis model. The analysis included histopathology of the joints, with a special emphasis on cartilage destruction, and measurement of mRNA levels for cytokines and inhibitors in synovial tissue and cartilage by reverse transcription–polymerase chain reaction (RT-PCR) technology. We demonstrated that the expression of CIA is under stringent control of endogenous IL-4/IL-10. Treatment with IL-4 alone was not effective, but IL-4 increased the suppressive action of IL-10 on both joint inflammation and cartilage destruction, even in established CIA. Apart from suppression of TNF/IL-1 mRNA levels, IL-4/IL-10 treatment resulted in up-regulation of the IL-1Ra/IL-1 balance. This latter finding suggests that IL-4/IL-10 treatment may offer a valuable alternative therapeutic approach to arthritis, apart from TNF/IL-1 inhibitors.

MATERIALS AND METHODS

Animals. Male DBA/1 Lac/J mice were obtained from Jackson Laboratories (Bar Harbor, ME). The mice were housed in filter-top cages, and water and food were provided ad libitum. The mice were immunized at the age of 9–10 weeks.

Materials. Freund's complete adjuvant (FCA) and *Mycobacterium tuberculosis* (strain H37Ra) were obtained from Difco (Detroit, MI). Lipopolysaccharide (LPS; *Escherichia coli* strain O111:84), ethidium bromide, rat Ig, and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM), Iscove's modified Dulbecco's medium (IMDM), DNA *Taq* polymerase, 100-basepair DNA marker, Trizol reagent, and agarose were obtained from Life Technologies (Breda, The Netherlands). GAPDH, IL-1β, IL-1Ra, TNFα, and tissue inhibitor of metalloproteinases (TIMP) primers were purchased from Pharmacia Biotech (Roosendaal, The Netherlands). Recombinant murine IL-3 (mIL-3) was obtained from R&D Systems Europe (Abingdon, UK). Recombinant murine IL-4 (10^7 units/mg) was a kind gift of Immunex (Drs. S. Gilles and M. Widmer, Seattle, WA). Purified recombinant mIL-10 (2.5×10^5 units/mg) was produced by Dr. A. Delvaux (Department of Medical Genetics-IRIBHN, Hospital Erasmus, Université Libre de Bruxelles, Brussels, Belgium). Murine IL-10 was cloned and expressed as previously described (32). The biological activity of IL-10 was verified in an LPS-mediated lethal-shock model (27).

IL-4 and IL-10 bioassays. For IL-4 determination, the IL-4–dependent CT.4S line was used (kindly provided by Dr. W. E. Paul [33]). Cells were washed 3 times and were seeded in duplicate or triplicate 0.1-ml cultures at 1×10^4 cells/well in DMEM, supplemented with 5% fetal calf serum (FCS), L-glutamine (2 m*M*), pyruvate (1 m*M*), 2-mercaptoethanol (2-ME; 0.05 m*M*), and gentamicin (50 μg/ml), and a 100-μl sample or standard was added. After 48 hours of incubation, 1 μCi of ^{3}H-thymidine/well was added; 16 hours later, CT.4S cells were harvested and ^{3}H-thymidine incorporation was determined.

The D36 mast cell line was used for IL-10 determina-

tion (34). D36 cells were cultured in IMDM supplemented with 5% FCS, L-glutamine (2 m*M*), 2-ME (0.05 m*M*), gentamicin (50 μg/ml), 10 units/ml of recombinant mIL-3, and 8 units/ml of recombinant mIL-4. For use as a bioassay, 2×10^3 cells were cultured in 0.1 ml of medium with the cytokine standard or the samples to be tested. After 24 hours, ^{3}H-thymidine (0.25 μCi/well) was added for another 24 hours, after which, the cells were harvested and the incorporated radioactivity was determined by scintillation counting. The IL-10 bioassay was performed in the presence of 8 units/ml of recombinant mIL-4.

Anti–mIL-4 and anti–mIL-10 antibodies. Hybridoma cells producing rat anti–mIL-4 antibodies (11B11) or rat anti–mIL-10 (JES5-2A5) were obtained from ATCC (Rockville, MD). Hybridoma cells (3×10^6) were injected into nude BALB/c mice, and after 3 weeks, ascites fluid was collected. Thereafter, Ig from ascites was isolated using a protein G column. Anti–mIL-4 antibodies (0.75 mg, which neutralized 1,500 units of mIL-4 in the CT.4S bioassay) and anti–mIL-10 (0.5 mg, which neutralized 25,000 units of mIL-10 in the D36 bioassay) were injected in a volume of 0.2 ml of phosphate buffered saline (PBS). As previously described, these doses were found to be effective in vivo (35,36). When the mice were treated with the combination of both antibodies, we injected 0.4 ml of PBS containing 1.25 mg of Ig. As a control, the same amount of normal rat Ig was injected.

Collagen preparation. Articular cartilage was obtained from the metacarpophalangeal joints of 1–2-year-old cows. Bovine type II collagen was prepared according to the method of Miller and Rhodes (37). Collagen was dissolved in 0.05*M* acetic acid (10 mg/ml) and stored at −70°C.

Immunization. Bovine type II collagen was diluted in 0.05*M* acetic acid to a concentration of 2 mg/ml and was emulsified in equal volumes of FCA (2 mg/ml of *M tuberculosis* strain H37Ra). The mice were immunized intradermally at the base of the tail with 100 μl of emulsion (100 μg of collagen). On day 21, the animals were given intraperitoneal (IP) booster injections of 100 μg of type II collagen dissolved in PBS.

Acceleration of CIA. In DBA/1 mice immunized with type II collagen and boosted on day 21, a gradual onset of CIA was noted beginning on day 28. CIA was scored visually at that time point. In general, the incidence of spontaneous arthritis ranged from 10% to 30% of the animals. Unless stated otherwise, mice without clear macroscopic signs of arthritis were selected at this stage and the onset of arthritis was accelerated by a single IP injection of 40 μg of LPS (21,38). This resulted in the onset of CIA within 3 days, and on day 35, full-blown arthritis was noted in the paws of >95% of the animals. The histopathology in the knee and ankle joints was comparable in mice with accelerated CIA and those with classic, spontaneous CIA. As described previously, administration of 40 μg of LPS to nonimmunized DBA/1 mice or mice immunized with a nonarthritogenic antigen did not result in any macroscopic or histologic abnormalities (23).

Assessment of arthritis. Mice were examined visually for the appearance of arthritis in the peripheral joints and severity scores (macroscopic score) were given as previously described (21,23). Mice were considered to have arthritis when significant changes in redness and/or swelling were noted in the digits or in other parts of the paws. At later time points, ankylosis was also scored macroscopically. The clinical severity of arthritis was graded on a scale of 0–2 for each paw, according to changes in redness and swelling, where 0 = no changes, 0.5 = significant swelling and redness, 1.0 = moderate, 1.5 = marked, and 2.0 = maximal swelling and redness and, later, ankylosis. The macroscopic score (mean ± SD) was expressed as a cumulative value for all paws, with a maximum possible score of 8. Knee joint inflammation was scored visually after skin dissection, using a scale of noninflamed/mild, marked, or severe inflammation. Scoring was done by 2 independent observers, without knowledge of the experimental groups.

Treatment of CIA with mIL-4 and mIL-10. To investigate the effect of mIL-4, mIL-10, and mIL-4/mIL-10 treatment of CIA, these cytokines were injected twice a day IP, starting at different time points after the onset of CIA. For both mIL-4 (29,57) and IL-10 (27), a total dose of 2,000 units/day was injected (0.2 μg of mIL-4 and 8 μg of mIL-10). As control, 8.2 μg of BSA was used.

To determine the effect of mIL-4 and/or mIL-10 on the early phase of CIA, treatment was started directly after the onset of arthritis (day 29 after immunization). For treatment of established CIA, mice were selected for the presence of arthritis and were randomly divided into groups of at least 10 mice. If unwanted skewing of groups was apparent after scoring, randomization was repeated. Therefore, each experimental group of mice had a similar macroscopic score before starting treatment with mIL-4 or mIL-10. Mice were treated for at least 7 days with mIL-4, mIL-10, or the combination of these cytokines.

At the end of anticytokine treatment, knee and ankle joints were isolated and processed for light microscopy. Tissue samples for mRNA measurements were also isolated at this time point.

Anti–mIL-4 and anti–mIL-10 treatment before the onset of CIA. On day 29 after immunization with type II collagen, DBA/1 mice that had no signs of arthritis were selected and divided into 4 groups of 15 animals. These groups were injected with either anti–mIL-4 (0.75 mg), anti–mIL-10 (0.5 mg), anti–mIL-4/anti–mIL-10 (1.25 mg), or rat Ig (1.25 mg). Injections were repeated on days 32 and 35. Mice were assessed daily for CIA until day 38. On day 39, mice were killed and sera were collected for antibody subclass examination.

Anti–IL-4 and anti–IL-10 treatment of established CIA. For elimination of endogenous mIL-4 and/or mIL-10 during established CIA, mice were treated with antibodies directed against these cytokines on days 32, 35, and 38. Treatment was started 4 days after LPS acceleration (day 28). In these particular experiments, 20 μg of LPS was used instead of the standard dose of 40 μg. We did this in order to achieve a moderate expression of CIA, which would allow for acceleration by treatment with anti–mIL-4 and/or anti–mIL-10. The same dosages of antibodies were used in these studies as described in the previous section.

Isolation of RNA. Mice were killed by cervical dislocation, and the patella and adjacent synovium were immediately dissected (39). Synovium biopsy tissue was taken from 6 of 10 patella specimens. Two biopsy specimens with a diameter of 3 mm were punched out, using a biopsy punch (Stifle, Wachtersbach, Germany): 1 from the lateral side and 1 from the medial side. Six patella specimens per experimental group were taken, and 3 lateral and 3 medial biopsy samples were

pooled to yield 2 samples per group. The synovium samples were immediately frozen in liquid nitrogen. Patellae were transferred to a 5% EDTA solution and kept on ice for 4 hours. Thereafter, the cartilage layer was stripped as previously described (21). This procedure does not affect mRNA isolation or amplification efficiency.

Total RNA from a pool of 10 cartilage samples from a particular group was extracted with 1 ml of Trizol Reagent, an improved single-step RNA isolation method based on the method described by Chomczynski and Sacchi (40). Synovium biopsy samples were ground to powder using a microdismembrator II (B. Braun, Melsungen, Germany). Total RNA was extracted in 1 ml of Trizol reagent in a manner similar to that used for cartilage samples.

PCR amplification. One microgram of synovial RNA and the total amount of cartilage RNA (pool of 10 cartilage layers) was used for RT-PCR. Messenger RNA was reverse transcribed to complementary DNA (cDNA) using oligo-dT primers and one-twentieth of the cDNA was used in 1 PCR amplification. PCR was performed at a final concentration of 200 μM dNTPs, 0.1 μM of each primer, and 1 unit of *Taq* polymerase (Life Technologies) in standard PCR buffer. The mixture was overlaid with mineral oil and amplified in a thermocycler (Omnigene, Hybaid, UK). Message for GAPDH, IL-1β, IL-1Ra, and TNFα was amplified using the primers described elsewhere (41–44). Primers for TIMP were designed using Oligo 4.0 and Primer Software.

Samples (5 μl) were taken from the reaction tubes after a certain number of cycles. PCR products were separated on 1.6% agarose and stained with ethidium bromide. The results are presented as the relative increase in mRNA expression compared with that in noninflamed control samples of normal DBA/1 mice. The relative increase in mRNA was calculated as follows: 1.9 (amplification factor [45]) to the power of n, where n is the difference in the number of cycles showing identical staining intensity for experimental and noninflamed control tissue. GAPDH levels were very consistent in all samples, not warranting any further correction of mRNA values for IL-1β, TNFα, IL-1Ra, and TIMP.

Determination of anticollagen antibodies. To investigate whether treatment of CIA with anti–mIL-4 and/or anti–mIL-10 enhanced type II collagen–specific antibody subtypes, we determined antibody titers on day 39. Antibodies against bovine type II collagen were determined with an enzyme-linked immunosorbent assay (ELISA). Titers of total Ig, IgG1, IgG2a, and IgE were measured. Briefly, ELISA plates were coated with 10 μg of bovine type II collagen. Thereafter, nonspecific bindings sites were blocked with 1% BSA solution. Serial 1:10 dilutions of the immune sera were added followed by an incubation with isotype-specific goat anti-mouse peroxidase (1:1,000; Nordic, Tilburg, The Netherlands) and substrate (5-aminosalicylic acid). Plates were read at 492 nm.

Histology. Mice were killed by ether anesthesia, and ankle and knee joints were removed and fixed for 4 days in 4% formalin. After decalcification in 5% formic acid, the specimens were processed for paraffin embedding (39). Tissue sections (7 μm) were stained with hematoxylin and eosin or Safranin O. Histopathologic changes were scored using the following parameters. Infiltration of cells was scored on a scale of 0–3, depending on the amount of inflammatory cells in the synovial cavity and synovial tissues. Proteoglycan depletion was determined using Safranin O staining. The loss of proteoglycans was scored on a scale of 0–3, ranging from fully stained cartilage to destained cartilage or complete loss of articular cartilage. A characteristic parameter in CIA is the progressive loss of articular cartilage. This destruction was graded separately on a scale of 0–3, ranging from the appearance of dead chondrocytes (empty lacunae) to complete loss of the articular cartilage. Histopathologic changes in the knee joints were scored in the patella/femur region on 5 semiserial sections of the joint, spaced 70 μm apart. For the ankle joint, we scored semiserial sections in a standardized region of the calcaneus. Scoring was performed on decoded slides by 2 observers, as described earlier (21,23).

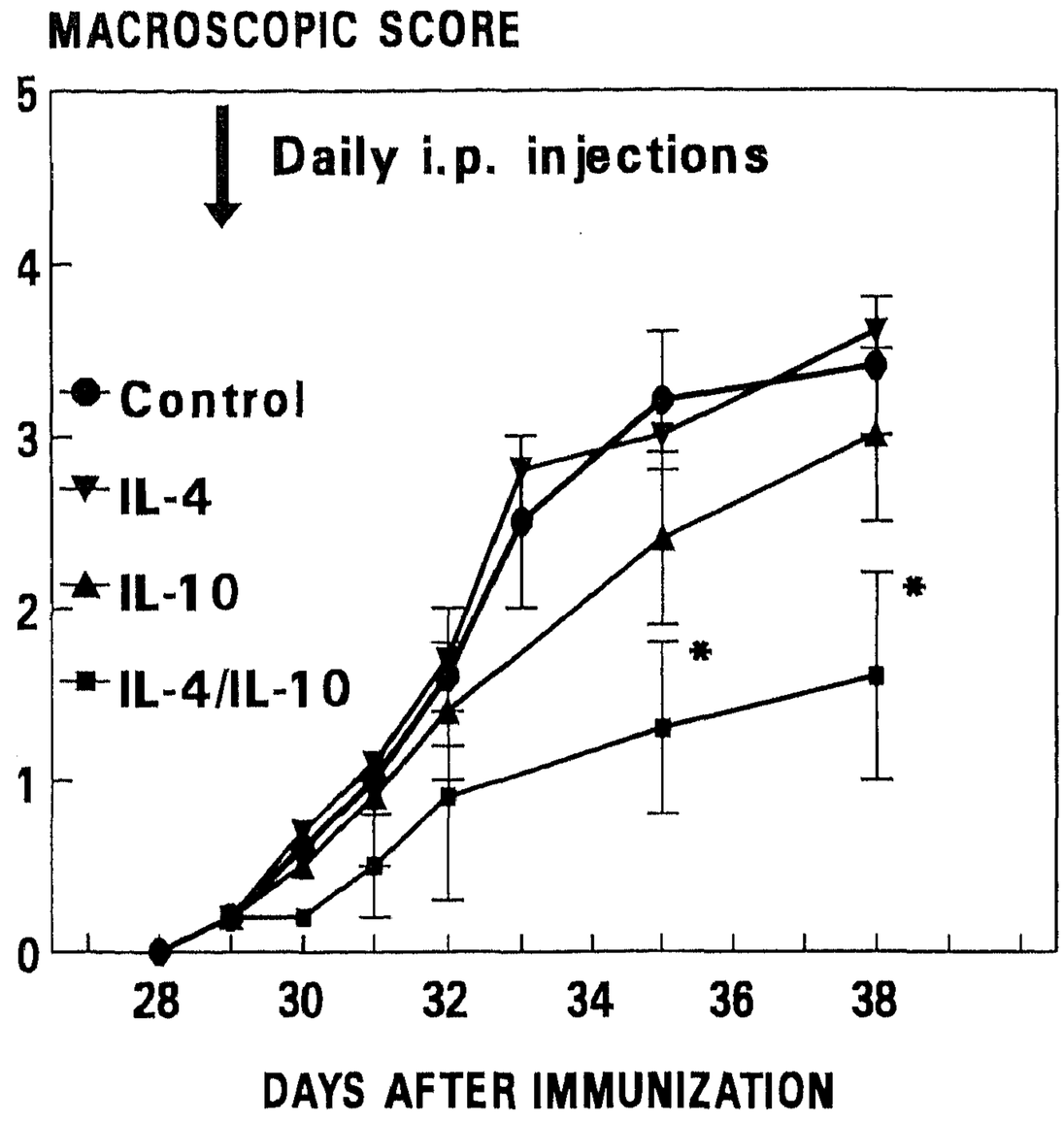

Figure 1. Early treatment of accelerated collagen-induced arthritis (CIA) with murine interleukin-4 (IL-4), IL-10, and IL-4/IL-10. Mice with the first signs of CIA (day 29) were divided into 4 separate groups of at least 10. Treatment with cytokines was started the same day. Cytokines were injected intraperitoneally (i.p.) twice a day at 1,000 units/injection; treatment continued for 9 days. Values are the mean ± SD macroscopic score for 3 experiments. * = $P < 0.05$ versus bovine serum albumin–treated controls, by Wilcoxon rank test.

Statistical analysis. Differences between experimental groups were tested using the Wilcoxon rank test, unless stated otherwise.

RESULTS

Modulation of CIA with mIL-4, mIL-10, or mIL-4/mIL-10 administered during onset. To investigate the effect of mIL-4, mIL-10, or the combination at the onset of CIA, mice were injected IP twice daily with 1,000 units of mIL-4, mIL-10, or mIL-4/mIL-10. Treatment was started on day 29 after the first immunization and

Table 1. Histology after murine IL-4/IL-10 treatment during the onset of collagen-induced arthritis*

Group	Start of treatment	Synovial infiltrates	Cartilage damage	Proteoglycan depletion	Day of killing
Knee joints					
Control	Day 29	1.0 ± 0.4	0.8 ± 0.4	2.0 ± 0.6	Day 38
mIL-4	Day 29	1.2 ± 0.5	0.7 ± 0.5	1.8 ± 0.5	Day 38
mIL-10	Day 29	1.4 ± 0.6	0.7 ± 0.5	2.1 ± 0.6	Day 38
mIL-4/mIL-10	Day 29	0.8 ± 0.7	0.2 ± 0.4†	1.2 ± 0.7†	Day 38
Ankle joints					
Control	Day 29	1.4 ± 0.4	0.4 ± 0.2	1.2 ± 0.8	Day 38
mIL-4	Day 29	1.2 ± 0.6	0.5 ± 0.3	1.3 ± 0.8	Day 38
mIL-10	Day 29	1.5 ± 0.5	0.4 ± 0.2	1.4 ± 0.7	Day 38
mIL-4/mIL-10	Day 29	0.7 ± 0.4	0.1 ± 0.1†	0.3 ± 0.4†	Day 38

* Start of treatment represents the number of days after intraperitoneal immunization of DBA/1 mice with bovine type II collagen lipopolysaccharide on day 28. Synovial infiltrates, cartilage damage, and proteoglycan depletion were scored on a scale of 0–3. The control group was treated with bovine serum albumin. Values are the mean ± SD of 2 experiments with at least 10 mice per group. mIL-4 = murine interleukin-4; mIL-10 = murine interleukin-10.
† $P < 0.01$ versus controls, by Wilcoxon rank test.

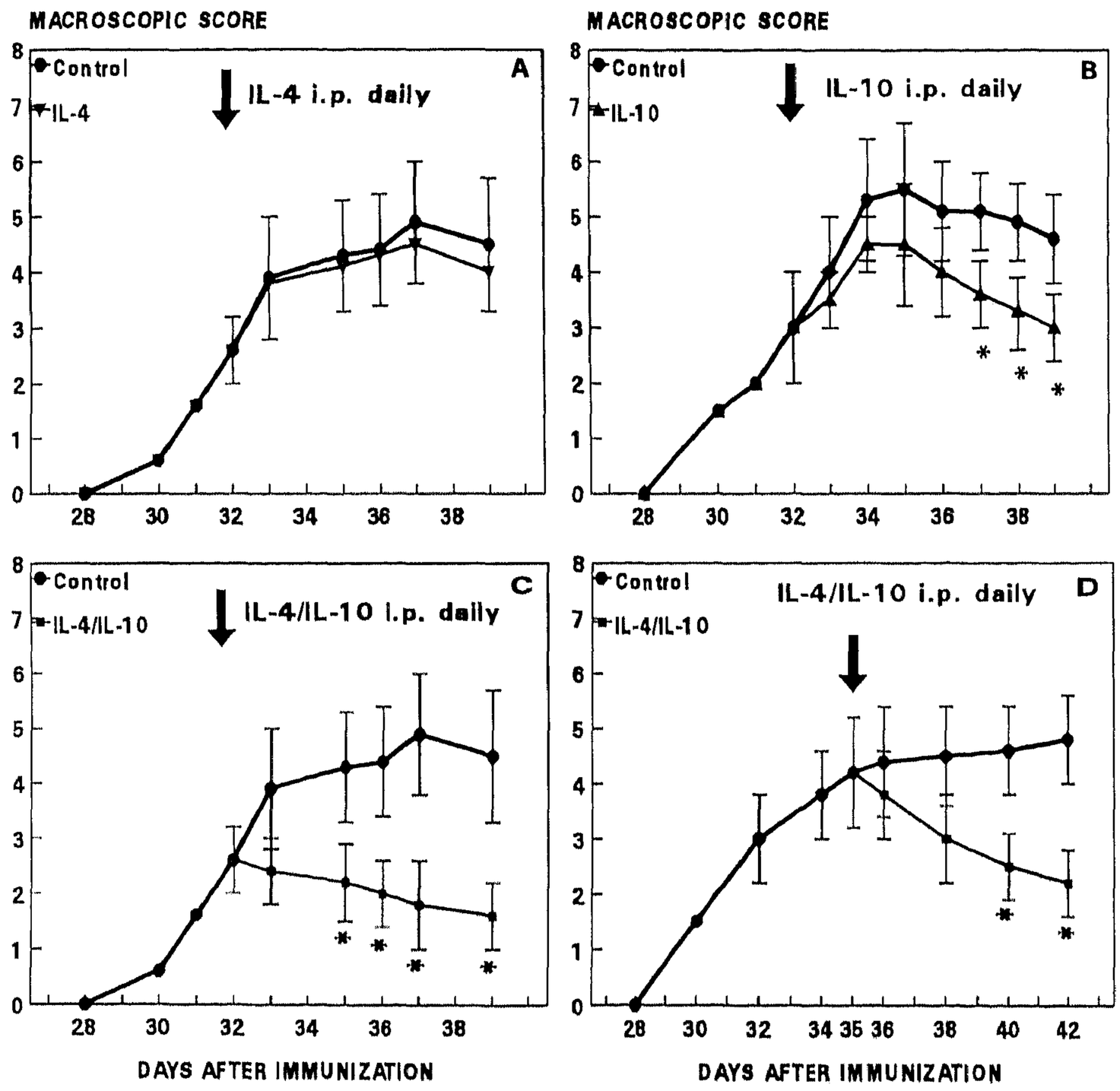

Figure 2. Effect of murine interleukin-4 (IL-4), IL-10, and IL-4/IL-10 treatment on established, accelerated collagen-induced arthritis (CIA). In separate experiments, the effects of treatment (arrows) beginning on day 32 of established CIA with **A,** murine IL-4, **B,** murine IL-10, or **C,** both murine IL-4 and IL-10, and, beginning on day 35, **D,** with both murine IL-4 and IL-10 were investigated. Cytokines were administered intraperitoneally (i.p.) twice a day at 1,000 units/injection; treatment continued for 7 days. Values are the mean ± SD macroscopic score of 3 experiments (10 mice per group). Scoring was performed daily by 2 independent observers who had no knowledge of the treatment group. At the end of the experiment, knee and ankle joints were dissected for histologic examination (see Table 2). * = $P < 0.05$ versus bovine serum albumin–treated controls, by Wilcoxon rank test.

Table 2. Histologic features after treatment of established collagen-induced arthritis with murine IL-4/IL-10*

Group	Start of treatment	Synovial infiltrates	Cartilage damage	Proteoglycan depletion	Day of killing
Knee joints					
Control	Day 32	1.5 ± 0.3	1.0 ± 0.4	2.5 ± 0.3	Day 39
mIL-4	Day 32	1.4 ± 0.3	1.2 ± 0.5	2.3 ± 0.6	Day 39
Control	Day 32	1.3 ± 0.3	1.1 ± 0.4	2.5 ± 0.3	Day 39
mIL-10	Day 32	1.2 ± 0.3	0.8 ± 0.5	1.9 ± 0.5	Day 39
Control	Day 32	1.2 ± 0.3	1.1 ± 0.3	2.4 ± 0.4	Day 39
mIL-4/mIL-10	Day 32	0.6 ± 0.3	0.5 ± 0.3†	1.3 ± 0.5†	Day 39
Ankle joints					
Control	Day 32	1.5 ± 0.4	0.8 ± 0.3	1.5 ± 0.2	Day 39
mIL-4	Day 32	1.3 ± 0.5	0.7 ± 0.3	1.6 ± 0.4	Day 39
Control	Day 32	1.6 ± 0.4	0.7 ± 0.2	1.3 ± 0.6	Day 39
mIL-10	Day 32	1.3 ± 0.3	0.6 ± 0.1	1.4 ± 0.5	Day 39
Control	Day 32	1.3 ± 0.5	0.9 ± 0.3	1.4 ± 0.5	Day 39
mIL-4/mIL-10	Day 32	0.6 ± 0.4	0.4 ± 0.2†	0.7 ± 0.3†	Day 39

* Start of treatment represents the number of days after intraperitoneal immunization of DBA/1 mice with bovine type II collagen lipopolysaccharide on day 28. Synovial infiltrates, cartilage damage, and proteoglycan depletion were scored on a scale of 0–3. The control group was treated with bovine serum albumin. Values are the mean ± SD of 3 experiments with at least 10 mice per group. mIL-4 = murine interleukin-4; mIL-10 = murine interleukin-10.
† $P < 0.01$ versus controls, by Wilcoxon rank test.

continued for 9 days. Murine IL-4 treatment had no effect on the course of the arthritis. Murine IL-10 treatment had a slight suppressive effect on the macroscopic arthritis score, but this did not reach statistical significance. However, in the mice treated with the combination of mIL-4/mIL-10, a marked reduction in the macroscopic joint score was noted (Figure 1). Increasing the dosages of mIL-4 and mIL-10 in the combined therapy (up to a concentration of 5,000 units/day) did not further enhance efficacy. When these higher dosages where given separately, mIL-4 remained without effect, whereas mIL-10 alone became suppressive as well. Moreover, in very recent studies using a highly purified preparation of mIL-10 (6×10^7 units/mg; Schering-Plough, Kenilworth, NJ), we reproduced these results with mIL-10 (data not shown).

In addition to visual scoring, we analyzed the histologic features in both knee and ankle joints on day 9 after the start of cytokine treatment. With this parameter, only the combination of mIL-4/mIL-10 showed a reduction in joint pathology (Table 1). Of importance, apart from the reduction in the amount of inflammatory cells, a significant decrease in cartilage damage and proteoglycan depletion was also seen.

Modulation of established CIA by mIL-4, mIL-10, and mIL-4/mIL-10. To extend the potential therapeutic effect to the later stages of the arthritis, the start of treatment was delayed to day 32 and day 35. Mice with marked arthritis on day 32 after immunization were treated with mIL-4, mIL-10, or mIL-4/mIL-10. Cytokines were injected IP twice a day in a concentration of 1,000 units per injection; this was continued for 7 days. Treatment of established CIA with mIL-4 did not improve the arthritis score, as can be seen in Figure 2A. In contrast, mIL-10 treatment of CIA resulted in a significant suppression of the macroscopic score of arthritis (Figure 2B). Interestingly, treatment of established CIA with the combination of mIL-4 and mIL-10 markedly ameliorated further expression of CIA (Figure 2C). Of importance from a therapeutic point of view, treatment of mice that had full-blown arthritis on day 35 with both mIL-4 and mIL-10 resulted in a significant suppression of the macroscopic score of CIA (Figure 2D).

Histology performed on day 39 (after 7 days of treatment) revealed that the combination of both mIL-4 and mIL-10 protected against cartilage destruction in the knee joints as well as the ankle joints (see Table 2 and Figure 3). Both cartilage damage and proteoglycan depletion were significantly reduced after treatment with mIL-4/mIL-10 for 7 days. Although mIL-10 suppressed the macroscopic score, no significant effect was found on joint histopathology. Murine IL-4 administration had no effect on the histologic parameters of cellular influx, cartilage damage, or proteoglycan depletion. This is consistent with the macroscopic findings after mIL-4 treatment of CIA.

In a repeat experiment, mice were treated for 7 days, starting on day 32, with the combination of mIL-4/mIL-10. Marked suppression was again found, confirming the above studies. On day 39, the mice were killed and mRNA was extracted from the synovial tissue and patellar cartilage of the knee. Cytokine mRNA