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L. A. B. Joosten, E Lubberts, P. Durez, Monique M. Helsen ...+3 more authors

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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

LEO A. B. JOOSTEN, ERIK LUBBERTS, PATRICK DUREZ, MONIQUE M. A. HELSEN, MARIKE J. M. JACOBS, MICHEL GOLDMAN, and WIM B. VAN DEN BERG

**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  $\alpha$  (TNF $\alpha$ ) 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 TNF $\alpha$  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- $\gamma$  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  $\alpha$  (TNF $\alpha$ ) 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  $\kappa$ B, 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

Leo A. B. Joosten, MSc, Erik Lubberts, MSc, Monique M. A. Helsen, Technician, Marike J. M. Jacobs, PhD, Wim B. van den Berg, PhD: University Hospital Nijmegen, Nijmegen, The Netherlands; Patrick Durez, MD, Michael Goldman, MD: Hospital Erasmus, Université Libre de Bruxelles, Brussels, Belgium.

Address reprint requests to Wim B. van den Berg, PhD, Professor of Experimental Rheumatology, Department of Rheumatology, University Hospital Nijmegen, P. O. Box 9101, 6500 HB Nijmegen, The Netherlands.

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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 TNF $\alpha$  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 TNF $\alpha$  and IL-1 by RA synovial tissue has been demonstrated (13). Recent clinical trials with neutralizing antibodies against TNF $\alpha$  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 TNF $\alpha$  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 TNF $\alpha$  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-1 $\beta$ , IL-1Ra, TNF $\alpha$ , 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 ( $2.5 \times 10^5$  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  $1 \times 10^4$  cells/well in DMEM, supplemented with 5% fetal calf serum (FCS), L-glutamine (2 mM), pyruvate (1 mM), 2-mercaptoethanol (2-ME; 0.05 mM), and gentamicin (50  $\mu$ g/ml), and a 100- $\mu$ l sample or standard was added. After 48 hours of incubation, 1  $\mu$ Ci 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 mM), 2-ME (0.05 mM), gentamicin (50  $\mu\text{g/ml}$ ), 10 units/ml of recombinant mIL-3, and 8 units/ml of recombinant mIL-4. For use as a bioassay,  $2 \times 10^3$  cells were cultured in 0.1 ml of medium with the cytokine standard or the samples to be tested. After 24 hours,  $^3\text{H}$ -thymidine (0.25  $\mu\text{Ci/well}$ ) 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 ( $3 \times 10^6$ ) 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 0.05M acetic acid (10 mg/ml) and stored at  $-70^\circ\text{C}$ .

**Immunization.** Bovine type II collagen was diluted in 0.05M 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  $\mu\text{l}$  of emulsion (100  $\mu\text{g}$  of collagen). On day 21, the animals were given intraperitoneal (IP) booster injections of 100  $\mu\text{g}$  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  $\mu\text{g}$  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  $\mu\text{g}$  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  $\pm$  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  $\mu\text{g}$  of mIL-4 and 8  $\mu\text{g}$  of mIL-10). As control, 8.2  $\mu\text{g}$  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  $\mu\text{g}$  of LPS was used instead of the standard dose of 40  $\mu\text{g}$ . 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.

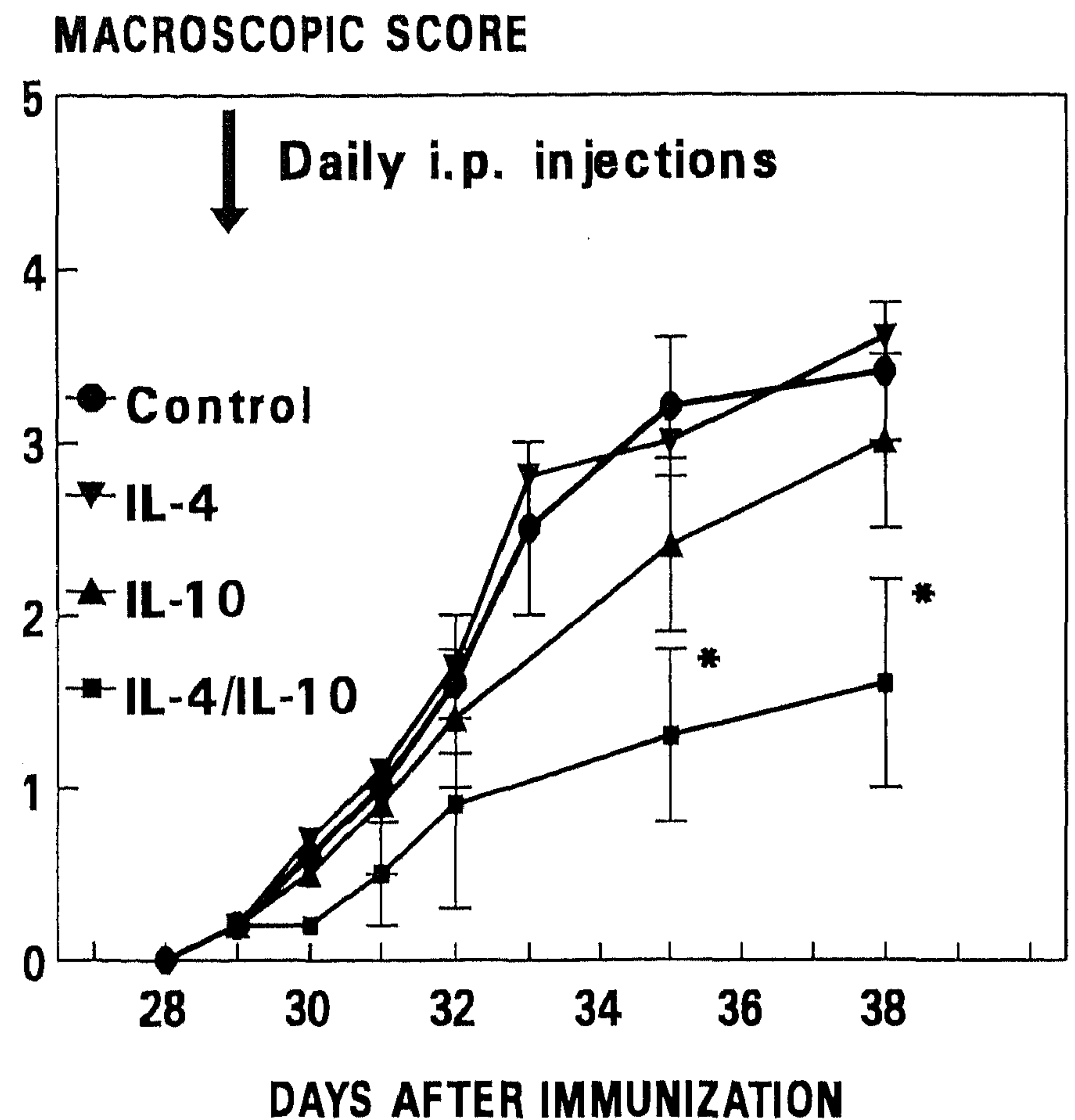
Total RNA from a pool of 10 cartilage samples from a particular group was extracted with 1 ml of Trizol Reagent, an improved single-step RNA isolation method based on the method described by Chomczynski and Sacchi (40). Synovium biopsy samples were ground to powder using a microdismembrator II (B. Braun, 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  $\mu$ M dNTPs, 0.1  $\mu$ M 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-1 $\beta$ , IL-1Ra, and TNF $\alpha$  was amplified using the primers described elsewhere (41–44). Primers for TIMP were designed using Oligo 4.0 and Primer Software.

Samples (5  $\mu$ l) 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-1 $\beta$ , TNF $\alpha$ , 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  $\mu$ g of bovine type II collagen. Thereafter, nonspecific binding 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  $\mu$ m) 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 proteogly-



**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  $\pm$  SD macroscopic score for 3 experiments. \* =  $P < 0.05$  versus bovine serum albumin-treated controls, by Wilcoxon rank test.

cans 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  $\mu$ m 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).

**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

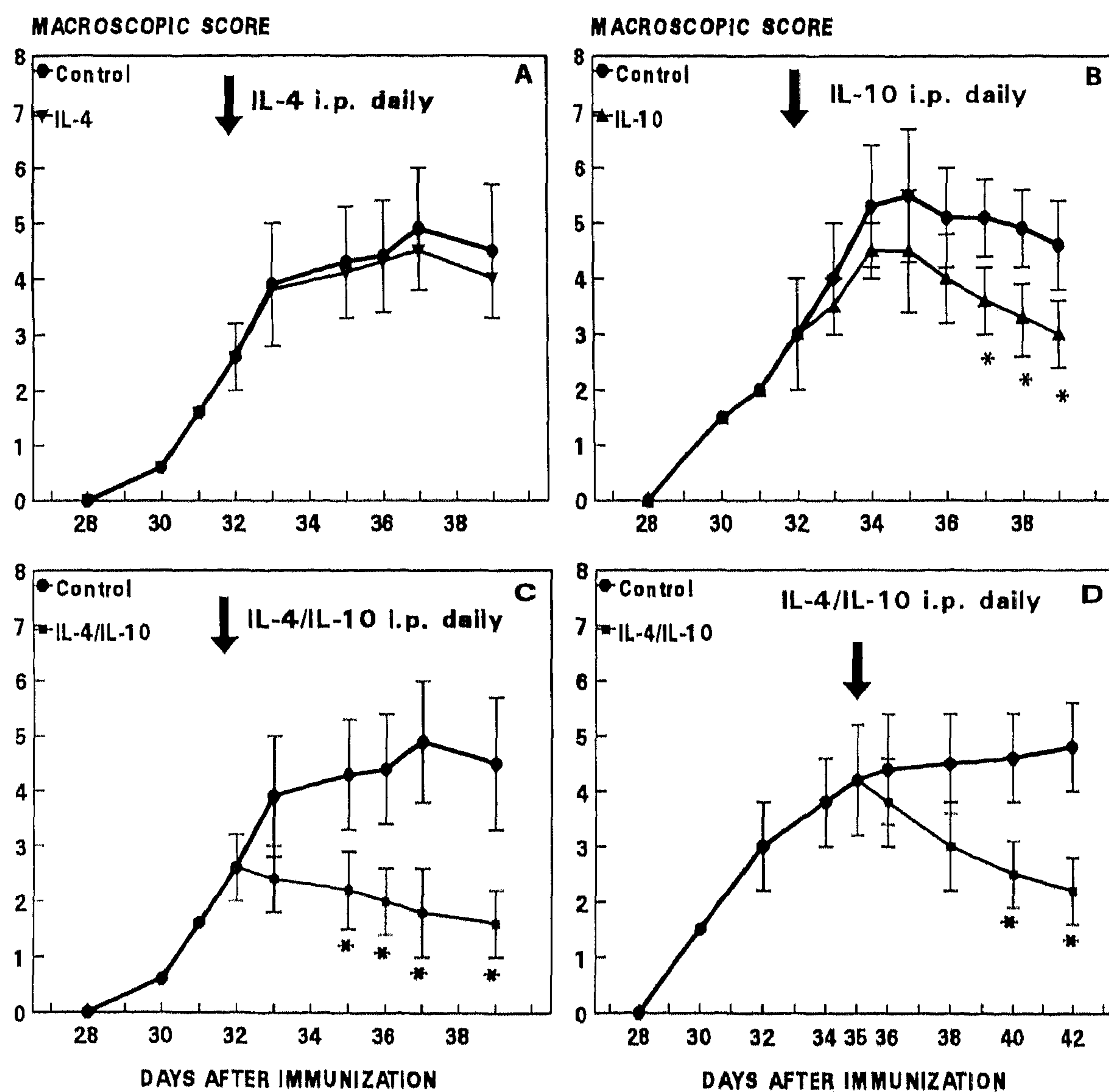


**Table 1.** Histology after murine IL-4/IL-10 treatment during the onset of collagen-induced arthritis\*

Group	Start of treatment	Synovial infiltrates	Cartilage damage	Proteoglycan depletion	Day of killing
<b>Knee joints</b>					
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
<b>Ankle joints</b>					
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
<b>Knee joints</b>					
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
<b>Ankle joints</b>					
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 were 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 ( $6 \times 10^7$  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.

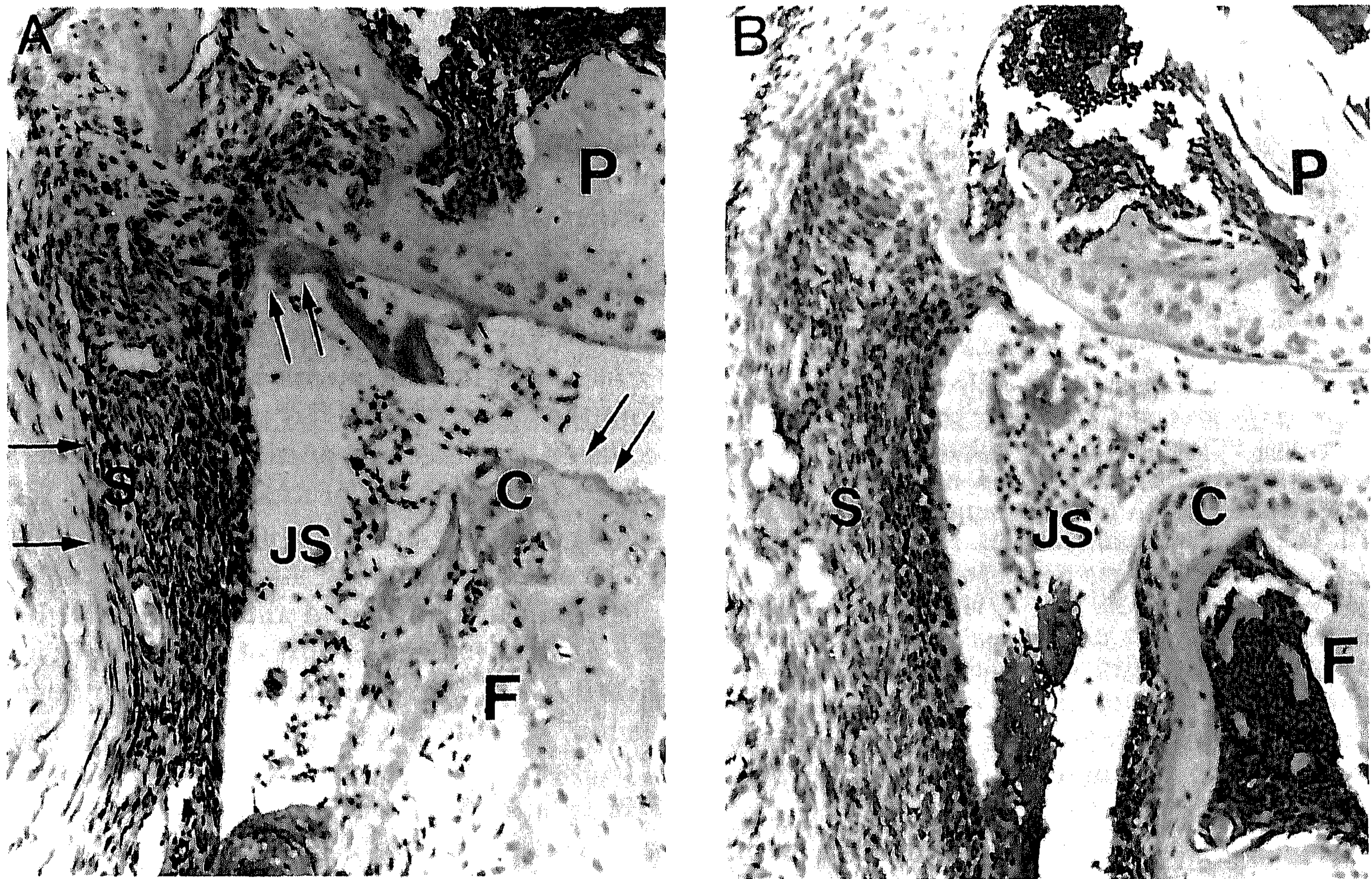
**Modulation of established CIA by mIL-4, mIL-10, and mIL-4/mIL-10.** To extend the potential therapeutic effect to the later stages of the arthritis, the start of treatment was delayed to day 32 and day 35. Mice with marked arthritis on day 32 after immunization were treated with mIL-4, mIL-10, or mIL-4/mIL-10. 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





**Figure 3.** Effect of late murine interleukin-4 (mIL-4)/mIL-10 treatment on cartilage destruction. **A**, Arthritic knee joint (day 39) of a control mouse treated with bovine serum albumin twice a day for 7 days. Note the enhanced infiltrate, severe cartilage surface disruption, and bone erosion (arrows). **B**, Knee joint of a mouse treated with mIL-4/mIL-10 for the last 7 days of study. **P** = patella; **S** = synovium; **JS** = joint space; **C** = cartilage; **F** = femur. Magnification  $\times 200$ .

levels (determined by RT-PCR analysis) are shown in Table 3. In inflamed synovium and patellar cartilage, there was a marked increase in IL-1 $\beta$  and IL-1Ra message compared with normal synovium and cartilage. Enhancement of mRNA for TNF $\alpha$  and TIMP was less

**Table 3.** Relative levels of cytokine mRNA in samples of inflamed tissue on day 39\*

Treatment	Tissue	IL-1 $\beta$	IL-1Ra	IL-1Ra:IL-1 $\beta$	
				ratio	TNF $\alpha$ TIMP
Control	Synovium	322	322	1	18 7
mIL-4/mIL-10	Synovium	25	322	13	6 7
Control	Cartilage	25	47	2	4 4
mIL-4/mIL-10	Cartilage	4	47	12	1 4

\* On day 39, synovium and cartilage samples were isolated as described in Materials and Methods. Values are the relative level of messenger RNA (mRNA) compared with normal (nonimmunized) DBA/1 mouse synovium or cartilage. The control group had arthritis. Values are the mean of 3 experiments in groups with collagen-induced arthritis. The polymerase chain reaction measurements of a particular mRNA sample were routinely repeated 3 times. The variation in the 3 repeat experiments never exceeded more than 2 cycles. IL-1 $\beta$  = interleukin-1 $\beta$ ; IL-1Ra = IL-1 receptor antagonist; TNF $\alpha$  = tumor necrosis factor  $\alpha$ ; TIMP = tissue inhibitor of metalloproteinases; mIL-4 = murine IL-4; mIL-10 = murine IL-10.

impressive. Treatment with mIL-4/mIL-10 clearly reduced the message for IL-1 $\beta$  and TNF $\alpha$  in both synovium and cartilage. Although IL-1Ra levels were not up-regulated, the ratio of IL-1Ra to IL-1 $\beta$  improved markedly after treatment with mIL-4/mIL-10, rising from 1 to 13 in the synovium. The same effect on the IL-1Ra:IL-1 $\beta$  ratio was found in cartilage. This is further illustrated in Figure 4. Remarkably, TIMP levels remained similar in the arthritic control, and the mIL-4/mIL-10-treated group, and this was found both in synovial tissue as well as in patellar cartilage.

**Involvement of mIL-4 and/or mIL-10 during onset of CIA.** To investigate whether endogenous mIL-4 and/or mIL-10 controlled the onset of CIA, we neutralized mIL-4 and/or mIL-10 in mice that had not yet developed CIA on day 29 after immunization. Mice were selected and divided into 4 groups of 15 mice. Groups then received either control rat Ig, anti-mIL-4, anti-mIL-10, or the combination (anti-mIL-4/anti-mIL-10) by IP injection. This treatment was repeated on days 32 and 35. Figure 5 shows a poor onset of arthritis in the selected control group. Treatment of such animals with anti-mIL-4 antibodies did not alter the course of the



	I	II	Cycli
GAPDH			20
IL-1 $\beta$			28
IL-1ra			32
TNF $\alpha$			38
TIMP			38

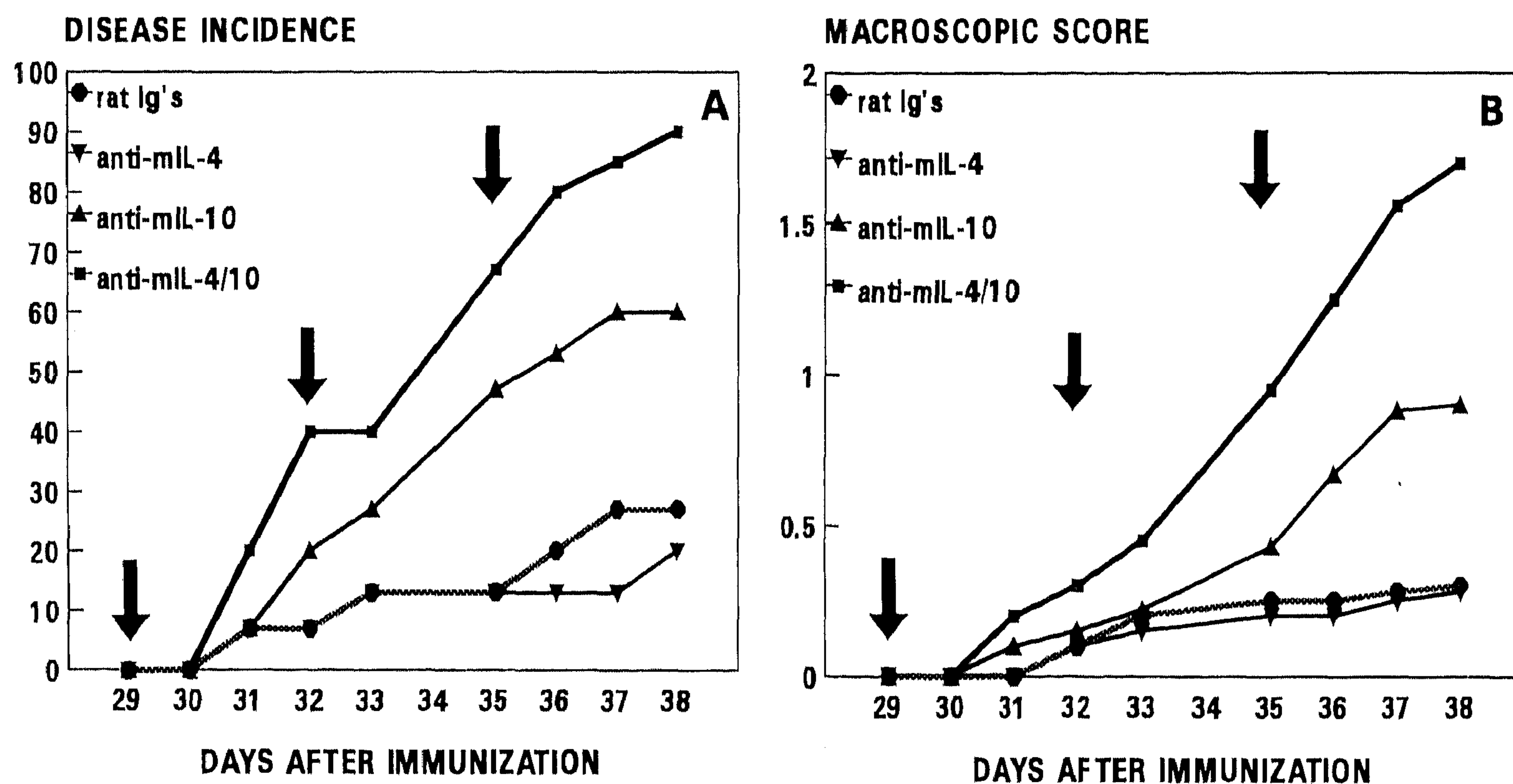
**Figure 4.** Polymerase chain reaction (PCR) analysis of patellar cartilage samples. Cartilage samples from 5 mice were pooled (10 samples) and RNA was extracted. PCR was performed as described in Materials and Methods. This experiment was repeated twice, with similar outcome. Lane I, Messenger RNA (mRNA) expression of GAPDH, interleukin-1 (IL-1), IL-1 receptor antagonist (IL-1ra), tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), and tissue inhibitor of metalloproteinases (TIMP) of arthritic control cartilage on day 39; lane II, mRNA expression in cartilage from mice treated for 7 days beginning on day 32 with mIL-4/mIL-10 (2,000 units/day). Cycli = the number of PCR cycles.

disease. However, elimination of mIL-10 during this phase markedly accelerated the expression of the disease, as determined by the incidence of arthritis as well as the macroscopic severity (Figures 5A and B). This indicated that mIL-10 is involved in suppressing the

expression of CIA. Neutralization of both mIL-4 and mIL-10 further accelerated the expression of type II CIA. These findings indicate that the onset of CIA is under control of IL-4 as well as IL-10. This proinflammatory effect of both anti-mIL-4 and anti-mIL-10 is further demonstrated in Table 4. Both the macroscopic score of knee joint inflammation (assessed after skin removal) and the histologic scores were increased after elimination of endogenous mIL-4 and mIL-10.

Anti-mIL-4 and/or anti-mIL-10 treatment before the onset of CIA did not induce a shift in type II collagen-specific isotype titers in the sera of arthritic mice. Antibody titers of total Ig (1:1,280), IgG1 (1:640), IgG2a (1:640), and IgE (1:320) were found in rat Ig-treated control mice and in mice treated with anti-mIL-4, anti-mIL-10, and anti-mIL-4/mIL-10.

**Involvement of mIL-4 and/or mIL-10 in established CIA.** To examine whether endogenous IL-4 and/or IL-10 also has a controlling role in established CIA, the expression of arthritis was first accelerated in a synchronized way with a moderate dose of LPS (20  $\mu$ g) on day 28. Separate groups of arthritic mice were then treated on days 32, 35, and 38 with either control rat Ig, anti-mIL-4, anti-mIL-10, or anti-mIL-4/anti-mIL-10.



**Figure 5.** Acceleration of collagen-induced arthritis (CIA) with anti-murine interleukin-4 (anti-mIL-4)/anti-mIL-10 antibodies. To examine the effect of elimination of mIL-4/mIL-10, separate groups of mice were treated with either anti-mIL-4 (0.75 mg), anti-mIL-10 (0.5 mg), or both before the onset (day 29) of CIA. As a control, 1.25 mg of rat Ig was injected. Administration of antibodies was repeated on days 32 and 35. Mice were assessed daily for arthritis; arthritis was deemed present when 1 or more digits was inflamed. Shown are A, the incidence and B, the macroscopic score. Values are the mean of 2 experiments (15 mice/group in the first; 10 mice/group in the second). Findings were consistent in the 2 experiments. After killing the mice on day 38, the knee joints were examined visually for joint inflammation (see Table 4A).



**Table 4.** Effect of anti-mIL-4, anti-mIL-10, or anti-mIL-4/anti-mIL-10 treatment on the expression of collagen-induced arthritis in knee joints\*

	Rat Ig	Anti-mIL-4	Anti-mIL-10	Anti-mIL-4/ anti-mIL-10
<b>Macroscopic score</b>				
None/mild, no. of mice	28	28	23	15
Marked, no. of mice	1	1	3	8
Severe, no. of mice	1	1	4	7
<b>Histopathology</b>				
% of joints with arthritis	17	20	37	70
Cell infiltrates, mean $\pm$ SD	0.1 $\pm$ 0.2	0.2 $\pm$ 0.5	0.6 $\pm$ 0.4	1.1 $\pm$ 0.5
Cartilage destruction, mean $\pm$ SD	0.1 $\pm$ 0.3	0.2 $\pm$ 0.3	0.2 $\pm$ 0.3	0.6 $\pm$ 0.3
Proteoglycan depletion, mean $\pm$ SD	0.1 $\pm$ 0.4	0.2 $\pm$ 0.5	0.7 $\pm$ 0.4	1.4 $\pm$ 0.4

\* Each group consisted of 15 mice that had no signs of arthritis when the first anticytokine treatment was given on day 29 (see arrows in Figure 5). Knee joints ( $n = 30$  per group) were assessed macroscopically on day 39 (after the skin was removed) by 2 independent observers, and the inflammation was scored as none, mild, marked, or severe. Joints were also examined histologically (score  $>0.25$  considered arthritic). Inflammatory cell infiltrates, cartilage destruction, and proteoglycan depletion were scored on a scale of 0–3. Anti-mIL-4 = anti-murine interleukin-4; anti-mIL-10 = anti-murine interleukin-10.

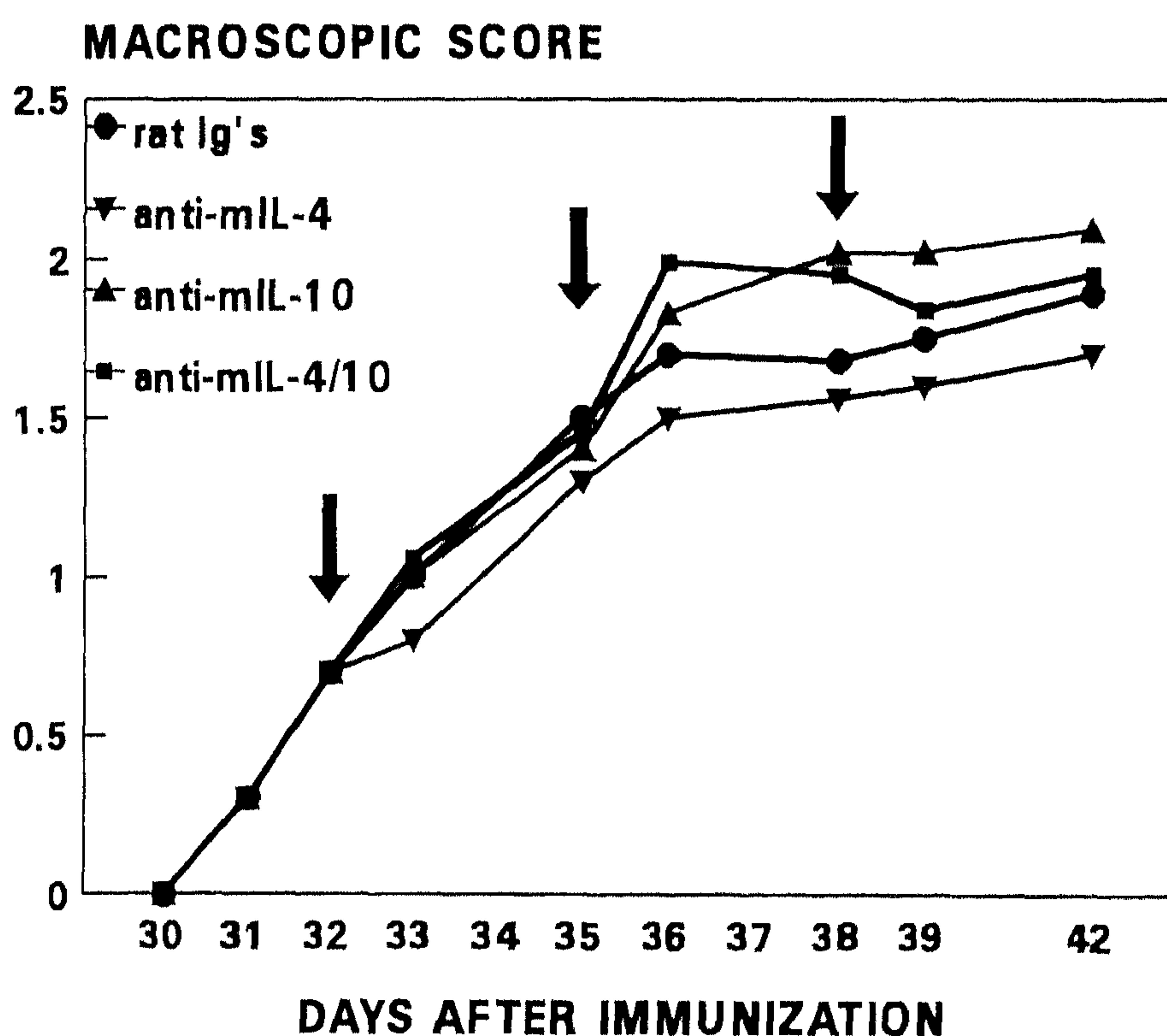
Neither treatment enhanced the expression of CIA compared with control Ig (Figure 6).

## DISCUSSION

Collagen-induced arthritis is a widely accepted model of rheumatoid arthritis, and understanding the regulation of the expression of this form of experimental

arthritis as well as insight into cytokine manipulation in an attempt to suppress established disease may provide better therapeutic approaches in patients with RA. The critical role of  $\text{TNF}\alpha$  and IL-1 in this model has been confirmed using neutralizing antibodies and IL-1Ra (21,22). In the present study, it has been clearly demonstrated that therapeutic benefit can also be achieved with combined treatment with IL-4 and IL-10, apparently by suppression of  $\text{TNF}/\text{IL-1}$  production and the concomitant shift in the IL-1Ra:IL-1 ratio. It is furthermore shown that IL-10 and, to a lesser extent, IL-4 are endogenous mediators in the prevention of disease expression.

There is ample evidence that IL-10 is an important down-regulator of a number of macrophage functions, including the production of  $\text{TNF}\alpha$  and IL-1 $\beta$ . IL-10 is abundantly present in joints with active RA (24), and in vitro studies with isolated synovial tissue revealed that  $\text{TNF}/\text{IL-1}$  production was markedly enhanced after anti-IL-10 treatment, whereas additional exogenous IL-10 was still able to further suppress this cytokine production (25). IL-4, but not IL-10, has been shown to enhance the production of IL-1Ra by RA synovial cells (26). The current findings make it clear that systemically administered IL-10, in conjunction with IL-4, markedly suppress established CIA. Although the effects were impressive, the degree of suppression was less than was achieved with anti-IL-1 $\alpha/\beta$  antibodies or IL-1Ra (21,22). This probably indicates that the suppression of IL-1 production with IL-4/IL-10 is not complete, which is also suggested by the data concerning the mRNA of the cytokines in the synovial tissue. Earlier studies from our group revealed that  $\text{TNF}$  is of importance at the



**Figure 6.** Effect of neutralization of endogenous murine interleukin-4 (mIL-4) and/or mIL-10 during established collagen-induced arthritis (CIA). Mice without signs of CIA on day 28 were injected with 20  $\mu\text{g}$  of lipopolysaccharide to accelerate the expression of CIA. Control rat Ig, anti-mIL-4, and/or anti-mIL-10 treatment was given on days 32, 35, and 38. Mice were assessed daily for arthritis. Values are the mean of 2 experiments (10 mice/group). For details, see Figure 5.



onset of CIA, whereas IL-1 is a pivotal cytokine both at the onset and at later stages of the disease (21,22). The critical dependence of IL-1 was recently confirmed by the total lack of onset of CIA in IL-1 $\beta$  knockout mice (46), although those animals showed normal reactions to a large range of inflammatory stimuli, including an undisturbed delayed-type hypersensitivity reaction (47).

There is suggestive evidence from *in vitro* studies that IL-4 and IL-10 have overlapping, but also distinct, activities. IL-4, but not IL-10, was shown to inhibit the production of leukemia inhibitory factor by rheumatoid synoviocytes (48). Moreover, up-regulation of IL-1Ra production by isolated monocytes has been found for both IL-4 and IL-10 (10). However, variable effects have been seen with synovial fluid macrophages, intact pieces of synovial tissue, and isolated synovial cells in terms of selective effects on IL-1Ra production as well as relative differences in the ability to suppress IL-1 and TNF production (26,49,50). Regulation is probably complicated by cytokine interplay between various cell types in inflamed tissue and may also depend on the maturation stage of the cells. In addition, it is now clear that IL-4 and IL-10 exert their suppressive effects on TNF/IL-1 production through different mechanisms, *i.e.*, by inhibited transcription and enhanced mRNA degradation, respectively (7). This fits with the cooperative effect of IL-4 and IL-10 in suppressing cytokine production by synovial cells (50). Our study is the first to show that IL-4 and IL-10 exert a clear cooperative effect in an arthritis model *in vivo*, and this appears to hold for both the early phase and the established phase of CIA.

Apart from suppression of macroscopic signs of inflammation, combined IL-4/IL-10 treatment also markedly reduced histologic scores, including the important parameter of cartilage destruction. CIA is an aggressive form of arthritis, showing rapid depletion of proteoglycans from the cartilage matrix as well as surface disruption. Both parameters markedly improved (Tables 1 and 2) after combined treatment with IL-4 and IL-10, but not with IL-4 or IL-10 alone, at a dosage of 2,000 units/day. We have done a few studies with higher dosages of IL-10 and have found more marked suppression of macroscopic swelling and synovial infiltrate with 5,000 units than with 2,000 units. This is in accordance with a recent study by Walmsley *et al* (31), who showed a clear dose dependency of the IL-10 effect and marked suppression with a dosage of 5  $\mu$ g (5,000 units) of IL-10 per day, starting directly after the onset of arthritis. In our studies, cartilage protection remained limited with IL-10 alone and never reached the marked effect noted with the combination.

Of interest, IL-4 may exert a direct protective

effect on cartilage destruction *in vitro*, exceeding the level of inhibition of TNF/IL-1 production. IL-4 has been shown to significantly reduce the cartilage proteoglycan depletion induced by TNF, IL-1, and LPS (51). Stromelysin levels were enhanced after IL-1, TNF, and LPS treatment, but IL-4 had no measurable effect on this parameter, leaving the mechanism of cartilage protection to be identified. Our analysis of cartilage mRNA levels after IL-4/IL-10 treatment *in vivo* showed reduced levels of TNF/IL-1 on the one hand and similar levels of IL-1Ra/TIMP on the other hand (Table 3), revealing a protective shift in the balance of control of destructive cytokines as well as degrading enzymes. The beneficial effect of IL-4/IL-10 directly on the chondrocyte may support the therapeutic value of such treatment, beyond that of therapy directed at elimination or blocking of TNF/IL-1.

IL-4 is known as a potent mediator in shifting the balance of Th1/Th2 cells and skewing the production of antibody subtypes. In earlier studies in lysozyme arthritis, with the dosage used in the present study, we have seen a marked shift in the production of antilysozyme antibodies (35). The current findings did not show a change in the subtypes of anticollagen antibodies, probably because IL-4/IL-10 treatment was given late (day 29) after a first immunization and a booster on day 21. Antibody levels are already very high at that stage, and the observation period is too short to expect a notable deviation. It further suggests that the effects of IL-4/IL-10 treatment on the arthritis were not related to major changes in anticollagen antibodies, although it cannot be excluded that crossreactive T cell responses to murine type II collagen are mounted at the destructive stage of the disease and may be skewed by IL-4.

IL-10 appears to be a natural suppressant of a number of inflammatory responses. IL-10 knockout mice showed normal immune responses and hematopoietic development at an early age, but older animals had chronic enterocolitis (52). Endotoxic shock and cutaneous inflammatory responses were enhanced in IL-10 knockouts (53,54). Likewise, blocking of endogenous IL-10 with neutralizing antibodies enhanced endotoxic shock, IgG immune complex-induced lung injury, and the severity of CIA (27,28,30). The latter study by Kunkel's group showed an increase in IL-10 at the mRNA and protein levels in joint tissues from the onset of arthritis, and accelerated expression of CIA was noted when anti-IL-10 treatment was started on day 24. This coincided with enhanced expression of the macrophage chemokines MIP-1a and MIP-2. We confirmed the anti-IL-10 effect, but also showed that the highest incidence and most severe arthritis was achieved after



elimination of both IL-10 and IL-4, although blocking of IL-4 alone did not influence arthritis expression. These observations are compatible with an important role of IL-4 and IL-10, in particular, in the control of arthritis expression, presumably by suppression of excessive production of TNF $\alpha$  and IL-1. The latter would fit with the earlier observations that a single dose of TNF $\alpha$  or IL-1, given shortly before the spontaneous onset of CIA, caused immediate expression of joint inflammation. Along the same line, our current findings indicated that antibodies against IL-4 and IL-10 did not further enhance CIA, which was already accelerated with LPS, probably because these mice already display high levels of LPS-induced TNF $\alpha$  and IL-1.

In contrast to the protective role played by IL-10 and IL-4 in CIA, these cytokines may have an opposite role in Th2-dependent processes. IL-10 was shown to exacerbate lupus-like autoimmunity in NZB  $\times$  NZW mice and pulmonary histopathology, induced with a pneumonia virus, could be abrogated by depletion of IL-4 and IL-10 (36,55). Moreover, IL-4 was recently shown to play an essential role in contact sensitivity skin reactions, transfer of delayed-type hypersensitivity by T cell lines, and T cell-mediated flares of lysozyme arthritis (56,57,35). In addition, it was suggested that IL-10 may have a pathogenic role in rheumatoid factor production in RA synovial tissues (24). Finally, in a model of LPS-induced uveitis, either suppressive or stimulating effects of IL-10 were found, depending on the dose of IL-10 applied (58). These observations underline the different roles in various disease processes and will complicate the therapeutic potential in diseases that are not fully understood. Since RA is considered a Th1 disease, with a dominant involvement of macrophage activation and excessive cytokine production, it seems likely that IL-4/IL-10 treatment may be of benefit in this disease and clinical trials are warranted.

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