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Adenoviral Vector-Mediated Overexpression of IL-4 in the Knee Joint of Mice with Collagen-Induced Arthritis Prevents Cartilage Destruction

Erik Lubberts,1* Leo A. B. Joosten,* Liduine van den Bersselaar,* Monique M. A. Helsen,* Andrew C. Bakker,* Joyce B. J. van Meurs,* Frank L. Graham,† Carl D. Richards,‡ and Wim B. van den Berg*

Rheumatoid arthritis (RA)1 is a chronic inflammatory joint disease, leading to cartilage and bone destruction. In this study, we investigated the effects of local IL-4 application, introduced by a recombinant human type 5 adenovirus vector, in the knee joint of mice with collagen-induced arthritis. One intraarticular injection with an IL-4-expressing virus caused overexpression of IL-4 in the mouse knee joint. Enhanced onset and aggravation of the synovial inflammation were found in the IL-4 group. However, despite ongoing inflammation, histologic analysis showed impressive prevention of chondrocyte death and cartilage erosion. In line with this, chondrocyte proteoglycan synthesis was enhanced in the articular cartilage. This was quantified with ex vivo 35S-sulfate incorporation in patellar cartilage and confirmed by autoradiography on whole knee joint sections. Reduction of cartilage erosion was further substantiated by lack of expression of the stromelysin-dependent cartilage proteoglycan breakdown neeopitope VDIPEN in the Ad5E1 mIL-4-treated knee joint. Reduced metalloproteinase activity was also supported by markedly diminished mRNA expression of stromelysin-3 in the synovial tissue. Histologic analysis revealed marked reduction of polymorphonuclear cells in the synovial joint space in the IL-4-treated joints. This was confirmed by immunolocalization studies on knee joint sections using NIMP-R14 staining and diminished mRNA expression of macrophage-inflammatory protein-2 in the synovium tissue. mRNA levels of TNF-α and IL-1β were suppressed as well, and IL-1β and nitric oxide production by arthritic synovial tissue were strongly reduced. Our data show an impressive cartilage-protective effect of local IL-4 and underline the feasibility of local gene therapy with this cytokine in arthritis. The Journal of Immunology, 1999, 163: 4546–4556.

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Therapy with this cytokine in arthritis. Our data show an impressive cartilage-protective effect of local IL-4 and underline the feasibility of local gene therapy with this cytokine in arthritis.

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In the present study, we examined the effects of local IL-4 over-expression, through a recombinant human type 5 adenovirus vector, in the knee joint of mice with collagen arthritis. We found that this treatment greatly protects against cartilage erosions, despite ongoing inflammation. The protective effect was associated with a reduction of PMNs in the synovial joint space, decreased NO synthesis, down-regulation of IL-1β, and a reduction of the MMP-3/TIMP disbalance in the synovium.

Materials and Methods

Animals

Male DBA-1/BOM mice were purchased from Bömholgdägg (Ry, Denmark). The mice were housed in filter-top cages. The mice were immunized between 10 and 12 wk of age. Water and food were provided ad libitum.

Adenoviral vectors

The recombinant replication-deficient adenovirus Ad5E1 mIL-4 was generated by homologous recombination after cotransfecting 293 cells with PACCMVMn1-4 and a virus-rescuing vector pAdBH10, as described (26). The empty recombinant replication-deficient adenovirus Ad5de170-3 was used as a control vector through the study. High titers of recombinant adenoviruses were amplified, purified, titrated, and stored, as described (27).

Materials

Freund’s complete adjuvant and Mycobacterium tuberculosis (strain H37Ra) were obtained from Difco (Detroit, MI). Bovine type II collagen (CII) was prepared as described (20). RPMI 1640 was obtained from Life Technologies (Breda, The Netherlands). ELISA plates (Maxisorb) were purchased from Nunc (Copenhagen, Denmark). The following mAb were used in the cytokine ELISAs: anti-murine IL-4 Abs (capture 18031D, detection 18042D) were purchased from PharMingen (San Diego, CA). Anti-murine IL-1β Abs (capture PM-425-B, detection MM-425-B) were obtained from Endogen (Cambridge, MA). Anti-murine IL-1Ra Abs (capture MAB480, detection BAFA480) were from R&D Systems (Minneapolis, MN). Streptavidin-polyperoxidase conjugate was obtained from CLB (Amsterdam, The Netherlands). Murine IL-4 Abs were a kind gift of Dr. S. Smith (Schering-Plough, Kenilworth, NJ). Murine IL-1β Ab was from R&D Systems. NIMP-R14 (rat IgG2b anti-mouse mAb) was kindly provided by Dr. M. Strath (National Institute for Medical Research, London, U.K.). Anti-VDIPEN IgG Abs were a generous gift of Drs. I. Singer and E. Bayne (Merck Research Laboratories, Rahway, NJ).

Induction of CIA

Bovine CII was diluted in 0.05 M acetic acid to a concentration of 2 mg/ml and was emulsified in equal volumes of Freund’s complete adjuvant (2 mg/ml of M. tuberculosis). The mice were immunized intradermally at the base of the tail with 100 μl of emulsion (100 μg of collagen). On day 21, mice were given an i.p. booster injection of 100 μg of CII diluted in PBS, and normally arthritis onset occurs at about day 28.

Study protocol

CIA was induced in male DBA-1 mice, as described above. Just before expected onset of CIA, mice were scored visually for the appearance of arthritis. Mice without macroscopic signs of arthritis in the paws were selected. Mice were anesthetized with ether, and a small aperture in the skin of the knee was performed for the intraarticular (i.a.) injection procedure. When absence of arthritis was confirmed in the knee joint, i.a. injections were performed with 10^6 or 10^7 PFU/μl of either an IL-4-expressing (Ad5SE1 mL-4) or an empty control (Ad5de170-3) recombinant human type 5 adenovirus vector or with saline. Seven days after the i.a. injection of the viral vector, mice were sacrificed by cervical dislocation after the viral vector, mice were sacrificed by cervical dislocation after injection of the viral vector, mice were sacrificed by cervical dislocation after injection of the viral vector, mice were sacrificed by cervical dislocation after injection of the viral vector, mice were sacrificed by cervical dislocation after injection of the viral vector, mice were sacrificed by cervical dislocation after injection of the viral vector.

Assessment of arthritis

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. Knee joint inflammation was scored visually after skin dissection, using a scale of noninflamed (0), mild (1), marked (1.5), or severe (2) inflammation. Scoring was done by two independent observers, without knowledge of the experimental groups.

Determination of IL-4, IL-1, β, and IL-1Ra protein

To determine the levels of IL-4, IL-1β, and IL-1Ra in patella washouts, patellae were isolated in a standardized manner from knee joints, as previously described (28). Patellae were incubated in RPMI 1640 medium with 0.1% BSA, gentamicin (50 μg/ml), and l-glutamine (2 mM) (200 μl/patella) for 1 h at RT. After supernatant was harvested, the IL-4, IL-1β, and IL-1Ra levels were measured by ELISA. Briefly, ELISA plates were coated with the capture Ab (3 μg/ml) by overnight incubation at 4°C in carbonate buffer (pH 9.6). Non-specific binding sites were blocked by 1% incubation at 37°C with 1% BSA in PBS/Tween. The supernatants from the patella cultures were tested by 3-h incubation at 37°C. The plates were then incubated for 1.5 h at 37°C with the biotinylated second Ab, followed by a 30-min incubation at 37°C with streptavidin-polyperoxidase conjugate. Bound complexes were detected by reaction with orthophenylenediamine and H2O2. Absorbance was measured at 492 nm using an ELISA plate reader (Titertek Multiscan MCC/340). The cytokine concentration in the samples was calculated as pg/ml using recombinant murine IL-4, IL-1β, or IL-1Ra as a standard. The sensitivity of the IL-4 and IL-1β ELISA is 10 pg/ml, and of the IL-1Ra ELISA is 100 pg/ml.

Isolation of RNA

Mice were sacrificed by cervical dislocation, and the patellae and adjacent synovium were immediately dissected (29). Synovium biopsy tissue was taken from 6 of 10 patellar specimens. Two biopsy specimens of a diameter of 3 mm were punched out, using a biopsy punch (Stifile, Wachtershau, Germany): one from the lateral side and one from the medial side. Three lateral and three medial biopsy samples were pooled to yield two samples per group. The synovium samples were immediately frozen in liquid nitrogen. Ten patella specimens per experimental group were taken. Patellae were transferred to a 5% EDTA solution and kept on ice for 4 h. Thereafter, the cartilage layer was stripped, as previously described (20). 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 (30). Synovium biopsy samples were ground to powder using a microdissection tool 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. mRNA was reverse transcribed to cDNA using oligo(dT) primers, and one-twentieth of the cDNA was used in one PCR amplification. PCR was performed at a final concentration of 200 μM dNTPs, 0.1 μM of each primer, and 1 U of Taq As mtq. Please verify, polymerase (Life Technologies) in standard PCR buffer. The mixture was overlaid with mineral oil and amplified in a thermocycler (Omniogene, Hybaid, U.K.). Message for GAPDH, IL-1, TNF-α, IL-1Ra, and inducible NO synthase was amplified using the primers described elsewhere (20). Primers for MMP-3, TIMP-1, macrophage-inflammation protein-2, and macrophage-inflammation protein-1 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.

Histology

Mice were sacrificed by cervical dislocation. Thereafter, whole knee joints were removed and fixed for 4 days in 10% formalin. After decalcification in 5% formic acid, the specimens were processed for paraffin embedding (31). Tissue sections (7 μm) were stained with hematoxylin and eosin (H&E) or Safranin O. Histopathological 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 (ex- cessive). Synovial tissue was scored as infiltrate (infiltrate). 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 chondrocyte (empty lacunae) to complete loss of the articular cartilage. Histopathological changes in the knee joints were scored in the patella/femur region on five semiserial sections of the joint, spaced 70 μm apart. Scoring was performed by two observers without knowledge of the experimental group, as described earlier (20).

For autoradiographic analysis, radiolabeled sulfate (50 μCi/mouse) was injected i.p. 2 h before dissection of the stifle joints. 35S-Sulfate was incorporated into the cartilage layer, but not the underlying subchondral bone, and its incorporation reflects newly synthesized proteoglycans (32). Sections (7 μm) were mounted on gelatin-coated slides, which were immersed in K5 emulsion (Ilford; Basildon, Essex, U.K.) and exposed for several weeks before being developed and stained with H&E.

Assessment of chondrocyte proteoglycan synthesis

Patellae (10 pieces), in a minimal amount of adjoining soft tissue, were placed in 2 ml RPMI 1640 medium with gentamicin (50 μg/ml), t-glutamine (2 mM), and 20 μCi [35SO4]sulfate. At the end of the 3-h incubation period, patellae were washed in saline three times, fixed in 4% formalin, and subsequently decalcified in formic acid (5%) for 4 h. Patellae were punched out of the adjacent tissue, and dissolved in 0.5 ml Luma solve (Omnilabo, Breda, The Netherlands). The 35S-Sulfate content of each patella was measured by liquid scintillation counting and expressed as cpm.

VDIPEN analysis

VDIPEN expression was analyzed as described (33). Briefly, sections were deparaffinized, rehydrated, and digested with chondroitinase ABC (0.25 U/ml 0.1 M Tris-HCl, pH 8) for 1 h at 37°C to remove chondroitin sulfate from the proteoglycans. Sections were then treated with 1% H2O2 in methanol for 20 min, and subsequently with 0.1% Triton in PBS for 5 min. After incubation with 1.5% normal rabbit IgG for 15 min, sections were incubated with NIMP-R14 (25–30 kDa epitope mainly present on neutrophils) Ab for 1 h. After incubation with the second rabbit anti-rat peroxidase Ab for 30 min, sections were incubated with AEC substrate in the dark by 37°C for 10 min. Thereafter, sections were stained with hematoxylin for 30 s. As a negative control, sections were incubated with normal rat IgG instead of NIMP-R14 Abs.

Assessment of nitrite levels in 48-h patella washouts

Patellae with adjacent synovium were isolated in a standardized manner from knee joints, cultured for 1 h in 200 μl RPMI medium at RT; thereafter, the culture supernatants were assayed for IL-4 by ELISA. The same doses of the Ad5del70-3 control vector i.a. injected in the left knee gave rise to undetectable levels of IL-4 (not graphed). No IL-4 levels were found in the circulation using 1.106 and 1.107 PFU doses of the control or IL-4-expressing vector (data not shown). Results are the mean ± SD of three mice per dose. The detection limit of the IL-4 ELISA is 5–10 pg/ml.

The concentration of NO2– (a stable breakdown product of NO) was determined by Griess reaction using NaNO2 standards (Merck, Darmstadt, Germany) in RPMI 1640 tissue culture medium with 0.1% BSA. Briefly, 100 μl of conditioned medium of 24 and 48 h was mixed with 100 μl of Griess reagent (0.1% naphthylethelenediamine dihydrochloride (Sigma, St.

Table I. Biological activity of IL-4 by the AdSE1mIL-4 vectora

<table>
<thead>
<tr>
<th>Dose (pfu)</th>
<th>Naive Mice Rabbit IgG</th>
<th>Naive Mice Anti-IL-4 Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Infiltratec</td>
<td>Exudatec</td>
</tr>
<tr>
<td>Ad5del70-3</td>
<td>1.106</td>
<td>0 (n = 8)</td>
</tr>
<tr>
<td>AdSE1mIL-4</td>
<td>1.107</td>
<td>0.8 ± 0.1 (n = 8)</td>
</tr>
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</table>

a Ad5del70-3 or AdSE1mIL-4 were given i.a. in the right knee joint of naive mice. Seven days later, mice were sacrificed by cervical dislocation and the knee joints were taken for histology.

b Rabbit anti-mouse IL-4 Ab (0.5 ml) were given i.p. at days 0, 3, and 6 to verify biological activity of IL-4. As a control, the same dose of rabbit IgG was used.

c Infiltration of cells was scored on a scale of 0–3, depending on the amount of inflammatory cells in the synovial cavity (exudate) and synovial tissue (infiltrate). Results are the mean ± SD per group.
Louis, MO), 1/1 diluted with 1% sulfanilamide (Sigma) in 5% H₃PO₄) in a flat-bottom microtiter plate (Costar, Cambridge, MA), and the OD at 545 nm was measured using an ELISA plate reader (Titertek Multiscan MCC/340).

**Statistical analysis**

Differences between experimental groups were tested using the Mann-Whitney rank sum test, unless stated otherwise.

**Results**

**Adenoviral vector-mediated IL-4 expression in the knee joint**

Different doses of Ad5E1 mIL-4 were intraarticularly injected into the right knee joint of naive and CII-immunized DBA-1 mice, and IL-4 protein levels were measured at day 7 in washouts of joint tissue. Considerable IL-4 levels were found after a single injection of 10⁷ PFU (Fig. 1), whereas roughly a 10-fold lower IL-4 level was seen after injection of 10⁶ PFU. No major differences were

**FIGURE 2.** Effects of Ad5E1 mIL-4 in the mouse knee joint with CII arthritis. Immunized DBA-1 mice were i.a. injected in the right knee joint with 1.10⁶ or 1.10⁷ PFU of Ad5E1 mIL-4 or Ad5del70-3, before onset of CIA was noted. Seven days after i.a. injection of the viral vector, mice were sacrificed by cervical dislocation and the skin of the knee joint was removed. The appearance of arthritis in the injected joints was assessed (A) and arthritis was scored for severity (B). Results are the mean ± SD of two experiments; in the first experiment, both doses of 1.10⁷ and 1.10⁶ PFU were tested with at least eight mice per group, and in a second experiment only the 1.10⁷ PFU dose was given, with at least 12 mice per group. *, p = 0.006 vs control group, by Mann-Whitney rank sum test.

**FIGURE 3.** Histological analysis of joint pathology after i.a. administration of Ad5E1 mIL-4 during onset of CIA. Immunized DBA-1 mice were i.a. injected in the right knee with 1.10⁶ or 1.10⁷ PFU of either Ad5E1 mIL-4 or Ad5del70-3 before onset of CIA was noted. Seven days after the i.a. injection of the viral vector, mice were sacrificed by cervical dislocation and the knee joints were taken for histology. Synovial infiltrates, exudates (A), and proteoglycan depletion (B) were scored on a scale of 0–3. For more detail, see Fig. 2. *, p = 0.002; #, p = 0.012 vs control group, by Mann-Whitney rank sum test.

**FIGURE 4.** Histological analysis of chondrocyte death (A) and cartilage erosion (B) after i.a. administration of 1.10⁷ PFU of Ad5E1 mIL-4 during onset of CIA. For more detail, see Fig. 3. Chondrocyte death and cartilage erosion were scored on a scale of 0–3. *, p < 0.001 vs control group, by Mann-Whitney rank sum test.

**Statistical analysis**

Differences between experimental groups were tested using the Mann-Whitney rank sum test, unless stated otherwise.

**Results**

**Adenoviral vector-mediated IL-4 expression in the knee joint**

Different doses of Ad5E1 mIL-4 were intraarticularly injected into the right knee joint of naive and CII-immunized DBA-1 mice, and IL-4 protein levels were measured at day 7 in washouts of joint tissue. Considerable IL-4 levels were found after a single injection of 10⁷ PFU (Fig. 1), whereas roughly a 10-fold lower IL-4 level was seen after injection of 10⁶ PFU. No major differences were
found between IL-4 levels in naive and CII-immunized mice. No detectable IL-4 was noted in washouts of normal knees or knee joints injected with a control vector (Ad5del70-3).

Local overexpression of IL-4 in the knee joint of naive mice gave rise to local cell influx at day 7. No increase in cell mass was noted using the same dose of Ad5del70-3. To verify biological

FIGURE 5. Protective effect of local IL-4 treatment on chondrocyte death and cartilage destruction in CIA. A, C, E, and G. Arthritic knee joint of a mouse 7 days after i.a. administration of $1.10^7$ PFU of Ad5del70-3 control vector. Note the infiltrate and chondrocyte death (A + C) (arrows), and severe cartilage surface disruption (E + G) (arrows). B, D, F, and H. Knee joint of a mouse 7 days after i.a. injection of $1.10^7$ PFU of Ad5E1 mIL-4. Note the enhanced infiltrate and almost complete prevention of chondrocyte death (B + D) and cartilage damage (F + H). P, patella; F, femur; S, synovium; JS, joint space; C, cartilage. A, B, E, and F, Original magnification ×100; C, D, G, and H, original magnification ×400. H&E was used in A–D, and Safranin O staining in E–H.
activity of IL-4 expressed by Ad5E1mIL-4, blocking experiments with anti-IL-4 were performed, resulting in prevention of cell influx (Table I).

Local IL-4 aggravates expression of collagen arthritis

DBA-1 mice were immunized with CII, and shortly before expected onset of collagen arthritis (day 25) a single injection of Ad5E1mIL-4, control vector, or saline was given in the right knee joint. Seven days later, a 100% arthritis incidence was noted in the right knee joints of the Ad5E1mIL-4 groups, both after injection of 10^7 or 10^6 PFU. In contrast, 89% and 50% incidence was seen in joints injected with 10^7 or 10^6 PFU control vectors, respectively. The latter was not different from the incidence observed after saline injection (Fig. 2). Of note, a single injection of 10^7 adenovirus did not induce joint inflammation in a knee joint of naive mice. This indicates that adenovirus itself can accelerate expression of collagen arthritis, whereas this expression is further increased when IL-4 is overexpressed.

Apart from the incidence, we also analyzed the severity of the arthritis. As seen in Fig. 2B, severity of arthritis is also markedly enhanced, even with the low dose of 10^6 PFU Ad5E1mIL-4. Furthermore, histologic analysis showed more inflammatory infiltrate in the synovial tissue and exudate in the synovial cavity in the low dose IL-4 group as compared with the control vector group (Fig. 3A).

Local IL-4 overexpression prevents chondrocyte death and cartilage erosion

Apart from the analysis of inflammatory aspects, the histologic knee joint sections were stained for proteoglycan content in the articular cartilage. Furthermore, semiserial sections were scored for the degree of chondrocyte death and cartilage surface erosions in the patella and femur region. Profound proteoglycan depletion was found in the control, arthritic group, and the depletion was not different in the IL-4 groups (Fig. 3B). However, despite the pronounced inflammation in the IL-4 groups, the degree of chondrocyte death and cartilage erosion was highly reduced (Fig. 4). A second experiment is shown in Fig. 4, in which the effect on the chondrocyte death and cartilage damage of a single injection of 10^7 PFU is shown. As in the first experiment, 100% arthritis incidence was noted in the right knee joint of the IL-4 group with an arthritis score of 2 ± 0.2 compared with 91% incidence in the adenoviral control group with an arthritis score of 1.8 ± 0.6. Again a strong reduction of the degree of chondrocyte death and cartilage erosion was found in the IL-4 group. Mean values were 61% and 77% reduced for these parameters in the high dose IL-4 group as compared with its respective adenoviral control group (Figs. 4 and 5).

In addition, the mice were ranked for individual scores of chondrocyte death and cartilage erosion in four subclasses (Table II). Whereas the majority of mice in the high dose control group were ranked as having severe chondrocyte death, none of the mice of the IL-4 group was in this category, whereas 13 of 21 were ranked as having no or mild chondrocyte death. When cartilage erosion was scored, the differences between IL-4 and control were even more impressive. All mice in the control group were ranked as moderate or severe, whereas all mice in the IL-4 group were nonerosive or mildly erosive.

Although the number of mice treated with the low dose (10^6 PFU) AdIL-4 is limited (n = 9), a similar trend of protection against severe damage was noted. Of the four of eight mice showing arthritis in the control group, three were classified with severe chondrocyte death and erosion (Table II). None of the nine arthritic
mice of the low dose IL-4 group ended up in this category, again proving that IL-4 provides protection against progression to major erosion.

Local IL-4 overexpression prevents VDIPEN neoepitope expression

VDIPEN neoepitope is a marker of metalloproteinase (MMP)-mediated cleavage of aggrecan, the major proteoglycan of articular cartilage, and earlier studies revealed that expression mainly occurred at sites and stages of advanced damage (33–35). To further demonstrate the protective effect of local IL-4, sections were stained for this neoepitope. As a typical example, VDIPEN was highly expressed at the edges of the patella and throughout the femoral cartilage in sections of control, arthritic mice with moderate erosions (Fig. 6). In contrast, VDIPEN staining was markedly reduced in the IL-4 group and only present at the margins of the cartilage.

In line with the reduced VDIPEN expression, we analyzed the mRNA expression of stromelysin, a major MMP able to induce the neoepitope cleavage site. RT-PCR measurements revealed a strongly reduced level of stromelysin in the synovial tissue (Table III). TIMP-1 was equally expressed in the synovium of control and

| Table III. Synovial and cartilage mRNA levels after Ad5E1mIL-4 treatment* |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | Synovium        | Cartilage       |                 |                 |                 |
|                 | Ad5del70-3      | Ad5E1mIL-4      | ▲ ▲             | Ad5del70-3      | Ad5E1mIL-4      | ▲ ▲             |
| IL-1B           | 26              | 31              | -5              | 25              | 28              | -3              |
| TNF-α           | 28              | 32              | -4              | 28              | 28              | 0               |
| IL-1Ra          | 26              | 27              | -1              | 26              | 26              | 0               |
| iNOS            | 32              | 33              | -1              | 34              | 35              | -1              |
| TIMP-1          | 21              | 21              | 0               | 24              | 22              | +2              |
| MMP-3           | 21              | 26              | -5              | 24              | 24              | 0               |
| MCP-1           | 26              | 26              | 0               | ND              | ND              | ND              |
| MIP-2           | 30              | 33              | -3              | ND              | ND              | ND              |

* Synovial and cartilage mRNA levels were determined by RT-PCR technology 7 days after the viral injection. Total RNA from a pool of 10 cartilage samples or 6 synovium biopsies from a particular group were pooled, as described in Materials and Methods. Values are the mean of two experiments. The PCR measurements of a particular mediator were routinely repeated three times. The variations in the two repeated experiments never exceeded more than two cycles.

Immunized DBA-1 mice were i.a. injected in the right knee joint with 1.10^7 pfu of Ad5E1mIL-4 or Ad5del70-3 before onset of CIA was noted. mRNA expression was determined 7 days after the viral injection.

▲ refers to the difference between the respective Ad5del70-3 and Ad5E1mIL-4 treated conditions.
IL-4 group, implying down-regulation of the MMP-3/TIMP-1 balance in the synovium of the IL-4-treated mice. Up-regulation of TIMP-1 was noted in the cartilage of the IL-4 group.

Effects of local IL-4 overexpression on chondrocyte metabolic function

The above studies revealed that IL-4 did prevent chondrocyte death. To investigate whether these chondrocytes are still metabolically active and produce proteoglycans, we measured $^{35}$S-sulfate incorporation in the whole patellar cartilage, ex vivo. As shown in Fig. 7, the $^{35}$S-proteoglycan synthesis is markedly enhanced in the IL-4 group as compared with the arthritic control, despite the fact that joint inflammation in these mice was undiminished. Because we noted above that local erosions and chondrocyte death were a major phenomenon in the control group, we also performed autoradiography on whole joint sections, after $^{35}$S-sulfate labeling in vivo. This showed variable but low metabolic activity of the chondrocytes in the control group. The IL-4 treatment induced a general increase in chondrocyte proteoglycan synthesis in chondrocytes throughout the whole cartilage (Fig. 8).

Local IL-4 overexpression reduces influx of granulocytes in the synovial joint space

Histologic analysis revealed differences in nature of the synovial infiltrate between the IL-4 group compared with the control vector group. In the control arthritic joint, numerous granulocytes adhered to the cartilage, whereas this was absent in the IL-4-treated joint (Fig. 9, A and B). Moreover, local IL-4 overexpression markedly diminished the influx of PMNs in the synovial joint space. This was confirmed by immunolocalization studies on knee joint sections using NIMP-R14 staining (Fig. 9, C and D) and diminished mRNA expression of macrophage-inflammatory protein-2 in the synovial tissue (Table III).

Table IV. Adenoviral vector-mediated IL-4 expression in the mouse knee joint suppresses local IL-1β and NO levels

<table>
<thead>
<tr>
<th>Arthritis Score</th>
<th>IL-1β (pg/ml)</th>
<th>NO (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad5del70-3</td>
<td>2.0 ± 0.06</td>
<td>309 ± 42</td>
</tr>
<tr>
<td>Ad5E1mIL-4</td>
<td>2.0 ± 0.05</td>
<td>75 ± 21</td>
</tr>
</tbody>
</table>

$^*$ Immunized DBA-1 mice with a booster injection on day 22 were i.a. injected in the right and left knee joint on day 26 with 1.10$^7$ pfu of Ad5E1mIL-4 or Ad5del70-3 before onset of CIA was noted. Seven days after the i.a. injection of the viral vector, patella washouts were taken and assayed for IL-1β and nitric oxide levels.

$^9$ Patellae with adjacent synovium were isolated in a standardized manner from knee joints and cultured for 1 h in 200 μl RPMI 1640 medium at room temperature; thereafter, IL-1β levels were measured in these culture supernatants using an IL-1β ELISA. Results are the mean ± SD of six patella washouts per group.

$^9$ Patellae with adjacent synovium were isolated in a standardized manner from knee joints and cultured for 24 h in 200 μl RPMI 1640 medium at 37°C; thereafter, medium was changed and the samples were incubated for another 24 h. NO levels were measured using Griess reagents. The results are the mean ± SD of the total nitric oxide levels of the first and the second incubation period.

$^*$, p = 0.002; †, p = 0.005 vs control group, by Mann-Whitney rank sum test.

Down-regulation of IL-1 and NO synthesis by local IL-4

Previously, we have shown the pivotal role of IL-1 in cartilage destruction in CIA (36–38). In addition, IL-1 is a key mediator in the regulation of the expression of MMP-induced neoepitope (33). Furthermore, it has been shown that IL-1 together with NO played an important role in the inhibition of the chondrocyte metabolic function of cartilage. It has been proposed that local IL-4 overexpression reduces influx of granulocytes in the synovial joint space, which in turn leads to a decrease in IL-1 and NO synthesis.
activity (39, 40). Furthermore, it has been shown that IL-4 up-regulates IL-1Ra, the receptor antagonist of IL-1 (9, 19). We therefore examined the effects of Ad5E1 mIL-4 on the local IL-1 expression in the synovium and cartilage and the local NO synthesis production. In addition, we analyzed the effects of local IL-4 on IL-1Ra and the balance IL-1α/IL-1β. RT-PCR revealed major down-regulation of mRNA expression of IL-1β in the synovium and also in articular cartilage (Table III). In line with this, pronounced suppression of IL-1β protein levels (76%) in the synovium was found (Table IV). Although no up-regulation of IL-1Ra mRNA levels (Table III) or protein levels (AdControl, 3831 ± 1329 pg/ml vs AdIL-4, 1959 ± 131 pg/ml) was found, the balance between IL-1Ra/IL-1β protein levels was slightly enhanced by local IL-4 treatment (IL-1Ra/IL-1β ratio for AdControl, 12 vs AdIL-4, 26). NO levels in the synovium were also decreased by Ad5E1 mIL-4 (Table IV). This indicates that the cartilage-protective effect of local overexpression of IL-4 in the synovium of mice with collagen arthritis might be due to suppression of local IL-1 production and subsequent reduction of MMP activity.

Discussion

This is the first study to demonstrate the major protection against cartilage erosion by local overexpression of IL-4 in the inflamed joint. Histologic analysis revealed almost complete protection against chondrocyte death and surface erosions of the articular cartilage, despite sustained, full-blown inflammation. This protective effect of local IL-4 was associated with down-regulation of IL-1, TNF, and NO in the synovium, whereas the MMP-3/TIMP-1 imbalance was improved.

Previously, we showed the pivotal role of IL-1 in cartilage destruction in murine collagen arthritis (36). Both IL-1α and IL-1β appear to be involved in cartilage destruction in Ag-induced arthritis, whereas dominant involvement of IL-1β was observed in collagen arthritis (37). This autoimmune model is driven by the combination of cellular and humoral immunity against cartilage CII and is characterized by rapid and severe erosions of cartilage and bone (41–44). The onset of CIA is dependent on IL-12, TNF, and IL-1, and under the stringent control of endogenous IL-10 (45, 20). The fact that the local IL-4 treatment strongly reduced IL-1 makes it likely that the protection against erosions is at least partly achieved through this pathway.

It has been shown that IL-4 up-regulates IL-1Ra (9, 19). In the present study, no direct up-regulation of IL-1Ra by local IL-4 overexpression was found. One explanation could be that IL-1Ra levels are high in articular tissue, as a feedback reaction to IL-1. When prolonged treatment with IL-4 highly reduces the IL-1 levels, this will result in significant down-regulation and this will outbalance the mere up-regulation by IL-4. Interestingly, because of the marked suppression of IL-1β, we found that the IL-1Ra/IL-1β balance was slightly up-regulated by local IL-4. Another explanation that could be involved is the fact that local IL-4 markedly prevents cell influx of granulocytes into the joint space. It is known that PMNs are important producers of IL-1Ra. The strong reduction of granulocytes in the exudate by local IL-4 may play an important role in the explanation that IL-1Ra is not up-regulated.

Protection against erosion was also substantiated by lack of VDIPEN neoeptipe expression in the cartilage of the IL-4–treated mice. Breakdown of aggrecan, the major proteoglycan of articular cartilage, predominantly occurs through two enzymatic pathways: aggrecanase attack, resulting in NITEGE neoeptiopes, and MMP-mediated degradation, resulting in VDIPEN neoeptiopes. Previous work from our group revealed that NITEGE expression is an early phenomenon in proteoglycan depletion and that VDIPEN epitope expression was mainly found at particular sites in the articular cartilage, associated with severe proteoglycan depletion and occurrence of chondrocyte death (J. B. J. Van Meurs, manuscript in preparation). Moreover, although inhibition of proteoglycan depletion was not always achieved with blocking of IL-1 in the various arthritis models, IL-1 blocking always fully prevented VDIPEN expression and late erosions. The present study further supports this link between erosions and VDIPEN expression. Although IL-4 treatment did not diminish proteoglycan depletion, it did prevent VDIPEN expression and surface erosions. It is known that IL-1 is a potent inducer of stromelysin (MMP-3) and that MMP-3 can induce the VDIPEN epitopes. The present study further revealed that MMP-3 mRNA levels were highly reduced by IL-4, compatible with a role of MMP-3 in erosion. It is not yet clear whether the reduction of IL-1 was indirectly responsible for the lowered MMP-3 levels or whether the IL-4 directly reduced the enzyme. In vitro studies have suggested that IL-4 is capable of direct reduction of these metalloproteinases (46, 47).

Apart from major reduction in cartilage erosions, we also noted an amelioration of the inhibition of chondrocyte proteoglycan synthesis, by direct measurement and autoradiography. Decreased [35]S-proteoglycan synthesis is a hallmark of events in the articular cartilage during joint inflammation, and IL-1 as well as NO, as a secondary mediator, were shown by us to be pivotal mediators in this process, in vivo (39, 40). The lower IL-1 and NO levels in the IL–4–treated mice are in line with these previous findings. Despite the recovery of synthetic activity, pronounced net proteoglycan loss was still observed in the cartilage of these mice, suggesting that there is overruling breakdown due to the inflammatory process, independent of IL-1 and NO. Of high interest, we found a similar uncoupling of NO dependence of proteoglycan synthesis and breakdown in recent studies in inducible NO synthase-deficient mice (40).

The prevention of cartilage damage in CIA was IL-4 dose dependent. Both the high and low dose of IL-4 vector significantly enhanced the expression of collagen arthritis, in line with potent proinflammatory potential of IL-4 (7). The best protection against cartilage erosion was obtained with the high dose, suggesting that substantial levels of IL-4 are needed to control erosion. Of interest, net proteoglycan depletion was not different between the two IL-4 dose groups, underlining uncoupling between depletion and erosion and suggesting that different mediators are involved in these processes.

Enhancement of synovial inflammatory mass by local IL-4 expression is a significant but not unexpected side effect. IL-4 has been shown to be a chemoattractant for macrophages and fibroblast and inducer of fibroblast proliferation (48–50). Increased cell influx and tissue proliferation have been noted after local IL-4 overexpression in the pancreas, trachea, and liver (51–53). Recently, up-regulation of β integrin, VCAM-1, IL-6, and monocyte-chemotactic protein-1 was reported after exposure of lung fibroblast to IL-4 (54).

The inflammation of collagen arthritis is characterized by a florid exudate in the joint space, containing numerous amounts of granulocytes, and a progressive destruction of the articular cartilage. Apart from the high numbers of granulocytes in the joint cavity, the synovial tissue contains large numbers of macrophages and lymphocytes, but also in this compartment granulocytes are prominent in the first 2 wk after onset. The most characteristic feature of collagen arthritis is the aggressive attack of the inflammatory process at the articular cartilage. In this model, heavy sticking of granulocytes at the cartilage surface is a common finding. Granulocytes may play an active role in the cartilage destruction, linked to sticking to anti-CII immune complexes in the surface...
layers. IgS adherent to cartilage surfaces have been identified in rheumatoid joints (55, 56), which can provide an anchorage and trigger for granulocyte activation (57). Reactive oxygen species and proteolytic enzymes present in the PMNs can be released directly into the surface of the cartilage, thereby escaping inhibitors present in the synovial fluid (58). PMNs need this close contact to cartilage to inflict cartilage damage (59–61). In the present study, we found numerous granulocytes adhered to the cartilage layer in the control arthritis joint, whereas this was absent in the IL-4-treated joint. This indicates that the marked reduction of granulocytes (PMNs) in the synovial joint space by local IL-4 may play an important role in the prevention of cartilage destruction.

The above paragraphs discussed various pathways of cartilage destruction in collagen arthritis. Apart from IL-1-mediated regulation of granulocyte influx, IL-1 is crucial in induction of latent enzymes in the articular cartilage, still needing further activation by other enzymes. Granulocytes may provide elastase, which is a potent activator of latent stromelysin. IL-4 can interfere with cartilage destruction at multiple levels. It reduces IL-1, it reduces stromelysin, and it prevents activation of latent stromelysin, through reduction of granulocyte influx and adherence to the cartilage surface. Although IL-4 enhances the inflammatory mass in the joint, cells do not show aggressive behavior. It is intriguing to note that joint inflammation in various patient groups can show a destructive and nondestructive phenotype. It is tempting to speculate that relative levels of IL-4 might make a difference.

It is known that IL-4 has a suppressive effect on Th1 activity and is a crucial factor in differentiation of naive T cells to the Th2 phenotype (12). In the present study, no substantial IL-10 expression was found in tissue washouts of the IL-4-treated group (data not shown). In contrast, preliminary studies on local IL-12 mRNA expression in the synovial tissue suggested marked reduction of this cytokine, implying that lower Th1 rather than enhanced Th2 expression in the synovial tissue suggested marked reduction of IL-4/IL-10 combination therapy than with IL-10 treatment alone.

In conclusion, this study represents the first demonstration of cartilage-protective effects of local IL-4 gene therapy in experimental arthritis, despite ongoing inflammation. It furthermore illustrates the feasibility of gene transfer. Apart from overexpression of inhibitors, probably asking for high and prolonged expression levels (38), the introduction of modulators such as IL-4 may provide a promising alternative to treat destructive arthritis. Our findings also underline the often suggested, but hardly proven concept that the balance of destructive and protective mediators determines the relative erosive nature of a given arthritis, rather than the bulk of the inflammatory mass. The main clinical problem of chronic arthritides is the destruction of cartilage and bone. Our data make it clear that IL-4 is a promising regulator of these elements.

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References


