Dual Role of IL-12 in Early and Late Stages of Murine Collagen Type II Arthritis

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IL-12 can promote Th1 responses, and early administration of IL-12 during immunization was shown to enhance expression of autoimmune collagen-induced arthritis (CIA). We now studied the impact of IL-12 at the stage of disease expression and during established CIA in DBA-1 mice. Accelerated onset and enhanced severity were provoked when i.p. injections of 100 ng of murine IL-12 (mIL-12) were given around the time of arthritis onset. Moreover, the onset of CIA could be ameliorated with anti-mIL-12 Abs, indicating that IL-12 is a pivotal mediator in the expression of CIA. In addition, the effect of anti-mIL-12 treatment was analyzed in established CIA. Continued treatment did not suppress established arthritis. Instead, these mice showed an impressive exacerbation of arthritis shortly after cessation of anti-mIL-12 treatment, indicative of impairment of endogenous control. Exaggerated disease was characterized by massive granulocyte influx and enhanced expression of IL-18 and TNF-α mRNA in the synovial tissue. Subsequently, we treated established collagen arthritis with recombinant mIL-12 for 7 days. Profound suppression of the arthritis score was noted, including reduced influx of cells and diminished cartilage damage. Tenfold enhanced levels of IL-10 were detected in sera of mIL-12-treated mice, and up-regulated mRNA levels of IL-10, IFN-γ, and IL-12 were measured in synovial tissue. Finally, the anti-inflammatory effect of IL-12 on CIA could be reversed by coadministration of anti-IL-10 Abs. This study indicates that IL-12 has a stimulatory role in early arthritis expression, whereas it has a suppressive role in the established phase of collagen arthritis. The Journal of Immunology, 1997, 159: 4094–4102.

IL-12 is a pleiotropic cytokine that is produced by APCs, macrophages, and B cells, and it promotes the growth of activated T cells and NK cells (1–4). IL-12 selectively generates the development of naïve T cells into Th1 cells and stimulates IFN-γ secretion by differentiated Th1 cells (5–7). IL-12 is efficiently induced in macrophages upon stimulation with LPS, several bacteria, and intracellular parasites (8–10). In LPS-induced lethal shock and generalized Shwartzman reaction, IL-12 plays a crucial role through the major induction of IFN-γ and TNF-α (11, 12). In general, IL-12 is considered to be a principal protective factor in bacterial infections, where it bridges innate resistance and Ag-specific immunity (13). On the other hand, IL-12 may unmask Th1-dependent autoimmune reactions and may be a crucial intermediate in the often-suggested link between bacterial infections and expression of autoimmune diseases (14). On the contrary, it controls excessive tissue pathology in allergy and parasitic infections by suppression of the Th2 response (15). Production of IL-12 by activated macrophages is enhanced by IFN-γ and is suppressed by IL-4 and IL-10 (16). More recently, it was demonstrated that IL-12 itself is a strong inducer of IL-10 production of T cells and macrophages in vitro, potentially providing an important negative feedback mechanism to prevent excessive activation and tissue pathology (17–21).

Collagen-induced arthritis (CIA) is widely accepted experimental model of polyarthritis. It can be induced in susceptible strains of mice and rats by immunization with heterologous type II collagen (CII), the major component of articular cartilage, and displays histopathologic features in common with human rheumatoid arthritis (RA) (22–24). The arthritis is dependent on the generation of a combination of anti-CII-Th1 cellular immunity and the production of anti-CII Abs, and full expression is best achieved upon immunization with CII in CFA. The Abs are essential elements in the onset of the arthritis. Complement-activating Abs bind to CII at the cartilage surface, with signs of acute inflammation, mild tissue damage, and further release of CII from the cartilage. Chronicity is then sustained by a T cell reaction in the synovial tissue. The monokines IL-1 and TNF enhance the autoimmune response to CII and exacerbate the arthritis (25). Furthermore, neutralization of IL-1 with anti-IL-1 Abs or IL-1Ra even suppressed fully established arthritis and ameliorated cartilage pathology, whereas TNF-α neutralization was less effective at that late, destructive stage (26–28). Recently, we demonstrated that expression of CIA is under stringent control of IL-4 and IL-10, in particular. Treatment with anti-IL-4/anti-IL-10 shortly before the onset of CIA accelerated disease expression. Moreover, IL-4/IL-10 treatment of established CIA resulted in clear suppression of the arthritis and prevented cartilage destruction (29).

The first studies of IL-12 in collagen arthritis were focused on early treatment and skewing of CII autoimmunity. IL-12 can replace mycobacteria in the immunization protocol and causes severe arthritis in DBA/1 mice when coadministered for 5 days with CII in ICF (30). A strongly enhanced anti-CII immunity was found. In contrast, high doses of IL-12 given to mice immunized

1 Abbreviations used in this paper: CIA, collagen-induced arthritis; CII, type II collagen; RA, rheumatoid arthritis; IL-1Ra, IL-1 receptor antagonist; mIL-12, recombinant murine interleukin-12; PO, peroxidase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.
with CII in CFA were shown to inhibit the development of arthritis, and this was associated with reduced anti-CII Ab levels (31). In the present study we focused on the potential role of IL-12 at the onset of arthritis, around day 28 after primary and booster immunizations with CII on days 0 and 21. At that stage the mice already display anti-CII T cell immunity and high anti-CII Ab titters. Studies include in vivo treatment with either mIL-12 or neutralizing anti-IL-12 Abs from day 28, and we are the first to demonstrate that such IL-12 treatment can accelerate arthritis expression, whereas anti-IL-12 Abs were shown to prevent arthritis onset. The most striking observation was the fact that anti-IL-12 treatment was without effect when started in fully established CIA on day 35, yet caused impressive exacerbation shortly after cessation of anti-IL-12 treatment, indicative of impairment of endogenous control. Further studies with late IL-12 treatment and neutralization with anti-IL-10 revealed impressive IL-12-mediated IL-10 generation and suggest that IL-12 has a stimulatory role in arthritis onset, yet a controlling role in the established stage of collagen arthritis. This suggests the need for subtle tuning of IL-12-directed therapy in human arthritis.

Materials and Methods

Animals

Male DBA-1 Lac/J mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Male DBA-1/Bom mice were purchased from Bomhildglird (Ry, Denmark). The mice were housed in filter-top cages, and water and food were provided ad libitum. The mice were immunized between 9 and 10 wk of age.

Materials

CFA and Mycobacterium tuberculosis (strain H37Ra) were obtained from Difco Laboratories (Detroit, MI). LPS (Escherichia coli, 0111:B4), eubilimum bromide, rat Iggs, sheep Igs, and BSA were purchased from Sigma Chemical Co. (St. Louis, MO). Taq DNA polymerase, 100-bp DNA marker, TRIZol reagent, and agarose were obtained from Life Technologies (Breda, The Netherlands).

Goat anti-murine Ig-peroxidase (PO), goat anti-murine IgG1-PO, and goat anti-murine IgG2a-PO were obtained from Southern Biotechnology Associates, Inc. (Birmingham, AL). GAPDH, IL-1β, IL-1Ra, IL-4, IL-10, IL-12, IFN-γ, TGF-β, and TNF-α primers were purchased from Eurogentec (Seraing, Belgium). Recombinant murine IL-12 (3 X 10^6 U/mg) and purified sheep anti-murine IL-12 IgG were provided by Dr. S. Wolf, Genteco (Breda, The Netherlands). Recombinant murine IL-10 ELISA kits were obtained from R&D Systems Europe Ltd. (Abingdon, U.K.). The cytoscreen murine IFN-γ ELISA was purchased from BioSource (Camarillo, CA). Hybridoma cells (JESS-2A5) producing rat anti-murine IL-10 were obtained from American Type Culture Collection (Rockville, MD).

Collagen preparation

Articular cartilage was obtained from meta-carpophalangeal joints of 1- to 2-yr-old cows. Bovine CII was prepared according to the method of Miller and Rhodes. Collagen was resolved in 0.05 M acetic acid (10 mg/ml) and stored at -70°C (26).

Immunization

Bovine CII was diluted with 0.05 M acetic acid to a concentration of 2 mg/ml and was emulsified in equal volumes of CFA (2 mg/ml MT 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 boosted with an i.p. injection of 100 μg of CII dissolved in PBS.

Acceleration of collagen-induced arthritis

When DBA-1 Lac/J mice were immunized with CII and boosted on day 21, a gradual onset of collagen arthritis was noted from day 28. At this point mice were visually scored for having collagen-induced arthritis. In general, arthritis incidence ranged from 10 to 30% of these animals. Mice without clear macroscopic signs of arthritis were selected at this stage, and the onset of arthritis was accelerated by a single i.p. injection of 40 μg of LPS (27, 32). This resulted in the onset of CIA within 3 days, and on day 35 full-blown expression of arthritis was noted in the paws of >95% of the animals. The histopathology in knee and ankle joints was comparable in accelerated CIA and classical CIA. As described previously, administration of 40 μg of LPS to nonimmunized DBA-1 Lac/J mice or to mice immunized with a nonarthrogenic Ag did not result in any macroscopic or histologic abnormalities (33).

Immunization of DBA-1/Bom mice resulted in high disease expression of 80 to 90% around day 28. Therefore, LPS acceleration was not necessary in these animals.

Assessment of arthritis

Mice were examined for visual appearance of arthritis in peripheral joints, and scores for severity were given (arthritis score) as previously described (26–29). Mice were considered arthritic when significant changes in redness and/or swelling were noted in digits or in other parts of the paws. At later time points ankylosis was also included in the arthritis score. Clinical severity of arthritis was graded on a scale of 0 to 2 for each paw, according to changes in redness and swelling: 0 = no changes; 0.5 = significant changes; 1.0 = moderate changes; 1.5 = marked changes; and 2.0 = maximal swelling and redness, and later ankylosis. Arthritis score (mean ± SD) was expressed as the cumulative value for all paws, with a maximum of 8. More than 90% of the animals that expressed CIA in a particular ankle joint expressed arthritis in the knee joint of the same leg.

Treatment of CIA with mIL-12, anti-mIL-12, or anti-mIL-10 Abs

To evaluate the effect of mIL-12 on the onset of CIA, DBA-1 Lac/J mice without signs of arthritis on day 28 were treated for 5 days with 100 ng of mIL-12 or BSA as a control. The role of endogenous IL-12 during LPS acceleration of CIA was studied by anti-mIL-12 treatment. Two hundred micrograms of purified sheep anti-murine IL-12 Abs (34) or sheep Igs were injected i.p., starting 2 h before LPS injection on the days indicated in Results. DBA-1 Bom mice with established CIA on day 35 were selected and divided into groups of at least 10 mice with similar arthritis scores. Thereafter, mice were treated daily with 100 ng of mIL-12, starting on day 35 up to day 42. Anti-mIL-12 treatment of DBA-1 Bom mice with full-blown CIA was given i.p., on the days indicated in Results. To investigate whether the IL-10 was involved in IL-12-mediated suppression of full-blown CIA, mIL-12 (100 ng/day) and anti-mIL-10 (0.75 mg of JESS-2A5) (29, 35) were injected. As a control we injected 0.75 mg of rat Igs and 100 ng of BSA i.p.

RNA isolation

Mice were killed by cervical dislocation, immediately followed by dissection of the patella with adjacent synovium (36, 37). Two synovium biopsies with a diameter of 3 mm were punched out of each patella, using a biopsy punch (Stiefel, Wachtersbach, Germany); one from the lateral side, the other from the medial side. Six patella specimens per experimental group were taken, and three lateral and three medial biopsies were pooled to yield two samples per group. The synovium samples were immediately frozen in liquid nitrogen. Synovium biopsies were ground to powder using a microdismembrator II (B. Braun, Melsungen, Germany). Total RNA was extracted in 1 ml of TRIZol reagent, an improved single step RNA isolation method based on the method described by Chomczynski and Sacchi (37, 38).

PCR amplification

One microgram of synovial RNA was used for reverse transcriptase (RT)-PCR. mRNA was reverse transcribed to cDNA using oligo(dT) primers, and 1/20th of the cDNA was used in one PCR amplification. PCR was performed using a final concentration of 200 μM dNTPs, 0.1 μM of each primer, and 1 U of Taq polymerase (Life Technologies) in standard PCR buffer. The mixture was overlaid with mineral oil and amplified in a thermocycler (Omnigene, Hybaid Ltd., Teddington, U.K.). Message for GAPDH, IL-1, IL-1Ra, IL-4, IL-10, IL-12, IFN-γ, TGF-β, and TNF-α was amplified using primers described previously (27, 39). Samples of 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. GAPDH levels were very consistent in all samples, not warranting any further correction of mRNA values for the cytokine of interest.

Blood analysis

Mice were bled after ether anesthesia, and blood was collected in 2-ml EDTA tubes, mixed well, and placed on 4°C. Thereafter, the levels of white blood cells, RBC, large unidentified cells, and hemoglobin and differentiation of the white blood cells were determined on a cytometric analyzer (Bayer/Technicon H2, Bayer, Tijdhrecht, The Netherlands).
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Determination of anti-collagen Abs

To investigate whether treatment of CIA with mIL-12 or anti-mIL-12 changed CII-specific Ab subtypes, we determined subtype Ab tiers. Abs against bovine CII were determined with an ELISA. The tiers of total Igs, IgG1, and IgG2a were measured. Briefly, ELISA plates (Maxisorb, Nunc, Copenhagen, Denmark) were coated with 10 μg of bovine CII. Thereafter, nonspecific bindings sites were blocked with 1% BSA solution. Serial 1/10 dilutions of the immune sera were added, followed by incubation with isotype-specific goat anti-mouse peroxidase (1/1000) and substrate (5-aminonosalic acid). Plates were read at 492 nm.

Histology

Mice were killed by ether anesthesia. Thereafter, 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 (26, 27, 36). Tissue sections (7 μm) were stained with hematoxylin and eosin. The Joss of proteoglycans was scored on a scale of 0 to 3, ranging from full 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 separately graded on a scale of 0 to 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 to 3, ranging from full stained cartilage to destained cartilage or complete loss of articular cartilage. Histopathologic changes in the knee joint, spaced 70 μm apart. Scoring was performed on decoded slides by two observers, as described previously (26, 27, 36).

Statistical analysis

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

Results

Role of IL-12 during the onset of CIA

DBA-1 Lac/J mice develop gradual onset of CIA after immunization with CII in CFA, ranging from 30 up to 90% on day 70. Early expression of CIA can be enhanced with a single i.p. injection of 40 μg of LPS at the time of onset on day 28 (Fig. 1A). To investigate the involvement of IL-12 in this acceleration, mice without signs of CIA were selected on day 28 and challenged with LPS. Treatment with 200 μg of purified sheep anti-mIL-12 Abs on days 28, 30, and 32, starting shortly after LPS injection, highly reduced CIA expression (Fig. 1A). Anti-mIL-12 treatment suppressed LPS-accelerated CIA expression to the level of onset of CIA in non-treated DBA-1 Lac/J mice. To further underline the accelerating role of IL-12, daily treatment with 100 ng of mIL-12 was given from day 28 to day 32. Enhanced disease incidence was evident within a few days (Fig. 1B).

In addition, we studied whether endogenous IL-12 is involved in the onset of CIA. As the onset of arthritis is rather variable in DBA-1 Lac/J mice, DBA-1 Bom mice were used, which normally show high arthritis incidence after CII immunization (80% incidence on day 37; Fig. 2A). Treatment with 200 μg of purified sheep anti-mIL-12 Igs on days 28, 30, and 32 suppressed both disease incidence and arthritis score of CIA (Fig. 2, A and B). Subsequent studies were all performed with DBA-1 Bom mice.

Role of IL-12 in established CIA

To investigate the involvement of IL-12 in established arthritis, treatment with neutralizing anti-mIL-12 Abs was started on day 35 and repeated on days 37 and 39. Unexpectedly, no suppression of arthritis severity was found. Instead, these mice showed an impressive exacerbation of arthritis, starting a few days after cessation of anti-mIL-12 treatment and suggesting that endogenous control of arthritis was impaired by anti-mIL-12 treatment. This experiment was repeated, and synchronized data are shown in Figure 3. In a third experiment the degree of exacerbation was less. As can be seen in Table I, the severity of the arthritis was already high in this group, probably explaining the lack of exacerbation. The exacerbation was confirmed with histology (Fig. 4). The number of inflammatory cells, in particular the relative number of granulocytes, was markedly increased. Cartilage damage was not significantly higher (Table II). Further proof of exaggerated disease was provided by enhanced IL-1β and TNF-α mRNA levels in the synovial tissue, whereas mRNA levels of IL-4, IL-10, IFN-γ, and TGF-β were unchanged (Table III).

Treatment of established CIA with rmIL-12

Since neutralization of endogenous IL-12 caused a marked exacerbation of arthritis, we wondered whether late treatment might have a therapeutic effect. DBA-1 Bom mice with established CIA on day 35 were treated for 7 days with 100 ng of rmIL-12. This resulted in gradual suppression of arthritis, reaching significance
on day 35 and divided into two separate groups of at least nine mice per group with roughly the same arthritis score. Anti-mIL-12 (200 μg) or control Igs were injected i.p. as indicated by arrows. Data are expressed as the mean ± SD percentage of the control (sheep Igs) arthritis score of two experiments (I and II, see Table I). * indicates p < 0.05, by Wilcoxon rank test, compared with the control group.

Table I. Exacerbation of CIA after anti-mIL-12 treatment

<table>
<thead>
<tr>
<th>Expt</th>
<th>Group</th>
<th>Arthritis Score</th>
<th>PMN Influx in Knee Joints</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Control</td>
<td>1.8 ± 0.8</td>
<td>2.0 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>Anti-mIL-12</td>
<td>1.8 ± 0.9</td>
<td>2.6 ± 1.2</td>
</tr>
<tr>
<td>II</td>
<td>Control</td>
<td>1.9 ± 1.2</td>
<td>2.8 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>Anti-mIL-12</td>
<td>2.0 ± 1.3</td>
<td>3.8 ± 1.4</td>
</tr>
<tr>
<td>III</td>
<td>Control</td>
<td>4.7 ± 1.2</td>
<td>4.2 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>Anti-mIL-12</td>
<td>4.6 ± 1.1</td>
<td>4.4 ± 1.0</td>
</tr>
</tbody>
</table>

* Mice with established CIA were selected at day 35 and divided into separate groups of at least nine mice with the same arthritis score. Three repeat experiments were performed. Sheep anti-mIL-12 Ab (200 μg) or control sheep Igs were injected at days 35, 37, and 39. In total, three identical experiments were performed.

b The number of knee joints is expressed in which marked influx of PMNs was found in synovial tissues or joint cavity. More than 90% of the animals expressed arthritis in both knee and ankle joint of the same leg. PMN = polymorphonuclear leucocytes.

c Arthritis score expressed as percentage of the control at day 52.

**Dominant role of IL-10 in IL-12-mediated suppression**

Plasma levels of IL-10 and IFN-γ were analyzed at the end of the 7-day IL-12 treatment (Fig. 8). Both parameters were markedly enhanced. To further underline the potential involvement of IL-10 up-regulation in the IL-12-mediated suppression, a final experiment was performed in which animals were treated with IL-12 as well as anti-mIL-10 Abs (Fig. 9). That study again confirmed the clear suppression of established arthritis with IL-12 treatment and, furthermore, made it clear that concomitant anti-mIL-10 treatment completely prevented the IL-12 effect. Histology of these groups showed suppression of inflammation only in the IL-12-treated mice (data not shown).

**Anti-collagen type II Ab titers and circulating leukocytes**

Involvement of shifts in anti-collagen type II Ab titers in exacerbations or suppressions of CIA seems unlikely. Anti-CII Ab levels are already high on day 35 of CIA, and neither mIL-12 nor anti-mIL-12 treatment caused major changes in the levels of anti-CII IgGs, IgG1, or IgG2A (data not shown).
Since it is known that high levels of IL-12 (1 μg/day) may have an impact on the bone marrow (40), differential counts of circulating leukocytes were performed. No major suppression in numbers of granulocytes, mononuclear cells, or lymphocytes were found (data not shown).

Discussion

IL-12 is an intriguing cytokine, playing an important role in early innate resistance to bacterial infections and the subsequent Ag-specific adaptive immunity. This is due to activation of NK cells and selective generation of Th1 responses. Likewise, IL-12 is an important defense mechanism against uncontrolled tumor growth.

As a dark side of this protective response, excessive IL-12 generation may unmask latent autoimmune disease. It was elegantly shown in the model of experimental allergic encephalomyelitis that the lack of encephalitis of Ig (myelin basic protein (MBP)-specific T cells from nonsusceptible mice could be restored upon exposure to IL-12 (14), and similar observations have been made in a range of autoimmune models (see below). The present study further identifies the potential of IL-12 to enhance arthritis expression in collagen arthritis-prone mice, but it also shows that IL-12 is a potent inducer of IL-10 and a down-regulator of the arthritic process in late stages of the disease.

CIA is an autoimmune model driven by the combination of cellular and humoral immunity against cartilage CIA. Upon immunization with CIA in CFA, the cytokine pattern in the lymphoid organs showed a dominant Th1 pattern (41). In line with this, IL-12 administered during immunization with CIA in IFA strongly enhanced Th1 responses, resulting in severe collagen arthritis (30). However, high doses of IL-12 were shown to suppress disease severity, associated with a marked reduction in CIA-specific Ab levels (31). Further analysis in susceptible and resistant mouse strains underlined the critical importance of high Ab levels. IL-12

| Table II. Effect of anti-IL-12 or mIL-12 treatment on knee joint pathology |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
|                             | Infiltrate                  | Cells in Joint Cavity       | Cartilage Damage            |
| Control                    | 1.2 ± 0.4                   | 0.5 ± 0.3                   | 1.8 ± 0.7                   | 1.6 ± 0.6                   |
| Anti-IL-12                 | 2.1 ± 0.5*                  | 1.3 ± 0.4*                  | 2.4 ± 0.9                   | 2.1 ± 0.5                   |
| Control                    | 1.8 ± 1.0                   | 1.7 ± 0.8                   | 2.0 ± 1.3                   | 2.2 ± 1.4                   |
| mIL-12                     | 0.7 ± 0.7*                  | 0.0 ± 0.0*                  | 1.9 ± 0.9                   | 1.4 ± 1.2                   |

* Anti-IL-12 (200 μg) treatment was started at day 28 (see Fig. 3) and repeated at days 30 and 32. As control, sheep IgG was injected. Histology was taken at day 52 after the first immunization with collagen.

** Mice were treated for 7 days with 100 ng mIL-12 started at day 35 (see Fig. 5). As control, we injected 100 μg BSA. Histology was performed at day 42 after immunization with collagen. Histology was scored on a scale ranging from 0 to 3 as indicated in Materials and Methods section.

*p < 0.05, Wilcoxon rank test. Values represent the mean ± SD of two identical experiments with at least nine mice per group.

| Table III. Synovial mRNA levels after treatment with anti-IL-12 or mIL-12 |
|-----------------------------|-----------------------------|-----------------------------|
|                             | PCR Cycles Day 52*          | PCR Cycles Day 42*          |
| Cytokine                    | Control                     | Anti-IL-12                  | Control                     | mIL-12*                     |
|                             | 8                           | 9                           | 1                           | 9                           | 9                           |
| IL-1β                       | 2                           | 2                           | 0                           | 2                           | 4                           |
| IL-1Ra                      | 4                           | 4                           | 0                           | 2                           | 8                           |
| IL-10                       | 2                           | 0                           | 2                           | 8                           |
| IFN-γ                       | 4                           | 4                           | 0                           | 9                           |
| TGF-β                       | 4                           | 4                           | 0                           | 6                           |
| TNF-α                       | 4                           | 6                           | 2                           | 4                           |

* Synovial cytokine mRNA levels were determined by RT-PCR technology. Synovium biopsies were taken at the end of the experiments. Synovium samples of six mice per group were pooled. Values are the mean of two experiments in groups with CIA. The PCR measurements of a particular cytokine were routinely repeated three times. The variations in the two repeated experiments never exceeded more than two cycles.

* * Number of PCR cycles in which gene product of interest was first detectable as compared with normal synovium.

* Cytokine mRNA expression at day 52 of CIA. Mice were injected three times with sheep IgG or with 200 μg sheep anti-IL-12 at days 35, 37, and 39. 

* A refers to the difference between respective control arthritus and anti-IL-12/mIL-12-treated conditions.

* Cytokine expression at day 42 in arthritic control and mIL-12-treated groups. MURINE IL-12 100 ng was given from day 35 up to day 42.

* a = antagonist.
was shown to enhance cellular, but not humoral, CII-specific Th1 responses in C57Bl and B10.Q mice and failed to induce arthritis (42). The critical importance of Abs in disease expression is compatible with our earlier observations of strong IL-1 dependence of both collagen arthritis and murine immune complex arthritis (26, 27, 43).

In the present study we further analyzed the role of IL-12 in the expression of joint inflammation, starting on day 28 after a first and a booster immunization. Anti-CII Ab levels were measured in various experiments, the absence of significant changes and made it unlikely that Ab shifts determined the arthritis outcome. We showed for the first time that late IL-12 treatment can still accelerate arthritis expression, whereas anti-IL-12 Abs, given shortly before onset, prevented both LPS-induced and classic onset of arthritis. This is probably related to IL-12-mediated IFN-γ production and generation of TNF and IL-1, mediators known to be of crucial importance in CIA onset (27, 44).

In other models of autoimmune inflammation, such as experimental allergic encephalomyelitis, diabetes in NOD (nonobese diabetic mice), experimental colitis, and glomerulonephritis, IL-12 played a potentiating role. In an adoptive transfer model of experimental allergic encephalomyelitis, IL-12 or anti-IL-12 treatment of T cells in vitro was shown to modulate subsequent disease expression (45). Likewise, early onset and severity of diabetes in NOD mice were markedly enhanced by in vivo IL-12 treatment, associated with massive influx of Th1 cells in islets (46). Furthermore, treatment of experimental colitis in mice with anti-IL-12 Abs abrogated the disease and improved the histopathologic aspects of the disease (47). Recent studies suggest that expression of collins in this model is due to loss of tolerance to resident intestinal flora, and this state can be restored with anti-IL-12 or IL-10 treatment (48). Finally, the expression of autoimmune disease in MRL/lpr mice was linked to the high capacity of this strain to produce IL-12 and the greater responsiveness of its macrophages to IL-12-mediated nitric oxide production (49).

**FIGURE 5.** Suppression of established CIA after IL-12 treatment. Arthritic DBA-1 B6 mice were divided into two separate groups of at least 10 mice on day 35. Murine IL-12 (100 ng) was injected i.p. once a day for 7 days. As a control, 100 ng of BSA was used. Data represent the mean ± SD arthritis score of two experiments. * indicates p < 0.05, by Wilcoxon rank test, compared with the control group.

**FIGURE 6.** Therapeutic effect of IL-12 treatment on joint pathology during established CIA. A, Arthritic knee joint (day 42) of the control (100 ng of BSA) group. Note the severe joint inflammation at this point of CIA. Histologic scores are 2.5 for infiltrate and 1.5 for cells in the cavity. B, Knee joint of a mouse treated for 7 days with 100 ng of mL-12. Clear suppression of inflammation and disappearance of granulocytes were found. Histologic scores were 0.5 for infiltrate and 0 for cells in the cavity. For details, see Figure 4.

The above set of data illustrates that IL-12 is a potentiating factor in a number of autoimmune diseases, including arthritis, and suggests the potential utility of anti-IL-12 Abs in patients. However, our present data on effects in fully established arthritis warrant great care with clinical application. They suggest a dual role of IL-12 in early and late arthritis, with dominant IL-12-mediated regulation of IL-10 production in late arthritis and a potential risk of exacerbation of arthritis upon IL-12 blocking. We showed impressive up-regulation of IL-10 in serum and synovial tissue after late IL-12 treatment, and the concomitant amelioration of arthritis is in line with earlier observations with IL-10 treatment (29, 50). Moreover, the IL-12 effect could be abrogated with anti-IL-10
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Intriguingly, IL-12 exacerbates, rather than suppresses, Th2-dependent pathology in IFN-γ knockout mice in the absence of endogenous IFN-γ (52).

Our data show that IL-12 enhances the onset of arthritis, probably related to enhanced IFN-γ and nitric oxide production and subsequent generation of TNF and IL-1. In late disease, IL-12 up-regulates itself as well as IL-10 and IFN-γ, without significant effects on IL-1 and TNF. Suppression of arthritis is probably due to IL-10, although it cannot be excluded that IFN-γ plays a role in this as well. Earlier studies in arthritis models showed variable effects of IFN-γ, with a tendency for suppression in established disease. It is known that repeated high dose IL-12 (1 μg/day) treatment may have unwanted side effects on bone marrow and lymph nodes. We have not seen such effects using daily treatment with 100 ng. Leukocyte counts in the blood were normal; the only change was some up-regulation of large unidentified cells, probably reflecting lymphoblasts.

The present study confirms a role for IL-12 in arthritis onset and, furthermore, identifies a regulatory role in late disease. It is now well accepted that TNF and IL-1, in particular, are key therapeutic targets in the treatment of human RA (53). Trials with anti-TNF provide great relief of symptoms, yet data on protection against ongoing cartilage destruction are under investigation. Trials with IL-1Ra are ongoing (54), and it is promising to see first data showing protection against joint erosions. In addition, new trials in RA patients are in progress with IL-4 and IL-10, focusing on the expected benefit of IL-1/TNF inhibition and additional up-regulation of cytokine and enzyme inhibitors as well as immunomodulation of RA orchestrating, with as yet unidentified Th1 responses. IL-12 would be a challenging target, with expected protection against onset or acute exacerbation of chronic arthritis upon IL-12 blocking. IL-12 has been detected in synovial fluid cells of RA patients (10). Moreover, recent studies in a murine arthritis, caused by Borrelia burgdorferi, showed a reduction in severity of arthritis with anti-IL-12 treatment (56).

Analysis of immune-mediated tissue injury in atherosclerotic plaques demonstrated that the degree of destruction is dependent on the critical balance of IL-12 and IL-10 (57). The present study urges great care with IL-12 elimination in established arthritis.

FIGURE 7. PCR analysis of synovial tissue. Synovium biopsies of six mice per experimental group were pooled, and RNA was extracted.

FIGURE 8. Plasma levels of IL-10 and IFN-γ after IL-12 treatment. Mice with full-blown CIA were treated for 7 days with either BSA (controls) or mlL-12 started on day 35 (see Fig. 5). Mice were bled on day 42, and plasma levels of IL-10 and IFN-γ were determined by ELISA. Values are expressed as the mean ± SD cytokine level of six mice per group of two experiments. * indicates p < 0.05, by Wilcoxon rank test, compared with the control.

FIGURE 9. Abrogation of the suppressive effect of IL-12 treatment on established CIA by coadministration of anti-mIL-10 Abs. DBA-1 Bm mice with established CIA were selected on day 33 and divided into four separate groups of 10 mice. Control mice received daily i.p. BSA (100 ng) and rat lgs (0.75 mg) as indicated by arrows. The IL-12 group was injected with 100 ng of mlL-12 and rat lgs as indicated by the arrows. The mlL-12/anti-IL-10 group received mlL-12 and anti-mlL-10 (0.75 mg). The anti-mlL-10 group was injected with BSA and anti-mlL-10. * Indicates p < 0.05, by Wilcoxon rank test, compared with the control.
linked to potential impairment of IL-10-mediated control. It is tempting to speculate that a combination treatment of anti-IL-12 with supplementation of IL-10 may provide optimal impact, but further insight into the regulation of IL-10/IL-12 balance in various stages of the disease is highly warranted.

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References

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