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J Immunol 2001; 167:1004-1013; ;
doi: 10.4049/jimmunol.167.2.1004
http://www.jimmunol.org/content/167/2/1004

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The American Association of Immunologists, Inc.,
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.

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IL-1-Independent Role of IL-17 in Synovial Inflammation and Joint Destruction During Collagen-Induced Arthritis[1]

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T cell IL-17 displays proinflammatory properties and is expressed in the synovium of patients with rheumatoid arthritis. Its contribution to the arthritic process has not been identified. Here, we show that blocking of endogenous IL-17 in the autoimmune collagen-induced arthritis model results in suppression of arthritis. Also, joint damage was significantly reduced. In contrast, overexpression of IL-17 enhanced collagen arthritis. Moreover, adenoviral IL-17 injected in the knee joint of type II collagen-immunized mice accelerated the onset and aggravated the synovial inflammation at the site. Radiographic and histologic analysis showed markedly increased joint destruction. Elevated levels of IL-1β protein were found in synovial tissue. Intriguingly, blocking of IL-1$\alpha\beta$ with neutralizing Abs had no effect on the IL-17-induced inflammation and joint damage in the knee joint, implying an IL-1 independent pathway. This direct potency of IL-17 was underscored in the unabated IL-17-induced exaggeration of bacterial cell wall-induced arthritis in IL-1$\beta^{-/-}$ mice. In conclusion, this data shows that IL-17 contributes to joint destruction and identifies an IL-1-independent role of IL-17. These findings suggest IL-17 to be a novel target for the treatment of destructive arthritis and may have implications for tissue destruction in other autoimmune diseases. *The Journal of Immunology,* **2001, 167: 1004–1013.**

Interleukin 17 is a recently discovered cytokine that is secreted by a restricted set of cells, whereas its receptor is ubiquitously expressed on many cell types (1–3). T lymphocytes, in particular the activated memory CD4$^+$ T cell subset (CD4$^+$CD45RO), are the major source of IL-17. Th1/Th0, but not Th2 subsets of CD4$^+$ T cell clones isolated from rheumatoid synovium produced IL-17 (4).

IL-17 may play an upstream role in T cell-triggered inflammation by stimulating stromal cells to secrete other cytokines and growth factors. It has the capacity to induce IL-6, IL-8, G-CSF, PGE$_2$, and the proinflammatory cytokines TNF-α and IL-1β (1, 5). This is compatible with the activation of NF-κB, which is known to regulate a number of gene products involved in cell activation and cell growth. Recently, it was found that TNFR-associated factor-6 was required in IL-17 signal transduction (6). A pathogenic role of IL-17 was found in organ allograft rejection, where it promotes maturation of dendritic cell progenitors and T cell proliferation (7). A further proinflammatory activity of IL-17 is demonstrated by its capacity to induce neutrophil recruitment through chemokine release (8) and stimulation of granulopoiesis (9).

Rheumatoid arthritis (RA)[3] is considered an autoimmune disease. It is a chronic systemic disorder of unknown etiology. The pathogenesis of RA is still unknown and seems to be multifactorial. Targeting the cytokine disbalance might represent a solid way to control this disease. RA is characterized by chronic inflammation of multiple joints and concomitant destruction of cartilage and bone. In the synovial membrane of the joints, cell-cell interactions between Th1 lymphocytes, monocytes, and synoviocytes underlie the enhanced production of the pivotal proinflammatory cytokines TNF and IL-1 (10). IL-17 production has been demonstrated in RA synovial tissue (11), and IL-17 enhances IL-1 mediated IL-6 production in vitro (12). IL-1 is a pivotal cytokine in cartilage destruction, and IL-17 shares these properties with IL-1, suggesting that infiltrated IL-17-producing Th1 cells may contribute to cartilage damage. In vitro studies identified that IL-17 suppresses matrix synthesis by articular chondrocytes through enhancement of NO production (13, 14). In addition, in vitro studies suggested a role of IL-17 in bone erosion. IL-17 induced the expression of receptor activator of NF-κB ligand (RANKL), which is a crucial factor in bone resorption (15, 16). These in vitro observations indicate that IL-17 may promote both joint inflammation as well as tissue destruction.

Murine collagen-induced arthritis (CIA) is a widely accepted arthritis model based on the generation of T cell and Ab-mediated autoimmune reactivity against a cartilage autoantigen, type II collagen (CII). The onset of arthritis is dependent on TNF-α, whereas IL-1 is crucial both in onset and propagation of arthritis (17). Th1 cells play a pivotal role in this model (18). The expression can be promoted by IL-12 administration during immunization as well as at the time of onset of arthritis. In addition, onset of arthritis could

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Received for publication November 15, 2000. Accepted for publication May 8, 2001.

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[1] This work was supported by Dutch Arthritis Association Grant NR 00-1-302.

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[3] Abbreviations used in this paper: RA, rheumatoid arthritis; CIA, collagen-induced arthritis; CII, type II collagen; SCW, streptococcal cell wall; i.a., intraarticular; PMN, polymorphonuclear cell; Ad, adenovirus; mu, murine.

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be blocked with anti-IL-12 Abs (19). In contrast, arthritis expression is under the control of IL-10 and can be suppressed by administration of the Th2 cytokines IL-4 and IL-10 (20). Recently, a major role of IL-15 was identified in CIA (21). This cytokine is a potent inducer of IL-17 (22).

So far, no studies have been done to identify the role of IL-17 in experimental arthritis models. In the present study, we examined the role of endogenous IL-17 in CIA with soluble IL-17 receptor protein (sIL-17R:Fc) and explored the amplifying activity of IL-17 by local overexpression of IL-17 in the joint. It was found that blocking of IL-17 significantly reduced CIA, including a clear suppression of joint damage. In contrast, local IL-17 overexpression promoted destructive arthritis. Of great interest, although IL-17 induced elevated levels of IL-1β, neutralization of IL-1 had no effect on this exaggeration, identifying an IL-1 independent role of IL-17. Our results suggest that IL-17-producing T cells can amplify arthritis, making it more destructive, and imply that similar processes may underlie tissue destruction in other autoimmune disorders.

Materials and Methods

Animals

Male DBA-1/BOM mice were purchased from Bomholdgärd (Ry, Denmark). A breeder pair of IL-1β-deficient mice was obtained from H. Zheng (Merck, Rahway, NJ) (23). As control animals, N/N (mixed 129sv $\times$ C57BL/6J background) were used. These animals were bred at our university breeding facilities in Nijmegen, The Netherlands. The mice were housed in filter-top cages. The mice were used between 10 and 12 wk of age. Water and food were provided ad libitum.

Materials

CFA and *Mycobacterium tuberculosis* (strain H37Ra) were obtained from Difco, Detroit, MI. Bovine CII was prepared as described previously (17). S. D. Lyman (Immunex, Seattle, WA) kindly provided murine IL-17R/human IgG1 Fc fusion protein (muIL-17R:Fc). RPMI 1640 was obtained from Life Technologies (Breda, The Netherlands). ELISA plates (Maxisorb) were purchased from Nunc (Copenhagen, Denmark). The following mAbs were used in the cytokine ELISAs: rat anti-murine IL-17 Abs (capture: MAB721) and a biotinylated goat anti-mouse IL-17 Ab (detection: BAF421) were purchased from R&D Systems (Minneapolis, MN). Streptavidin-polyperoxidase conjugate was obtained from CLB (Amsterdam, The Netherlands). Recombinant muIL-17 was obtained from R&D Systems. Rabbit anti-murine IL-1α,β polyclonal Abs were prepared in our own laboratory (24).

Adenoviral vectors

AdCMVmIL-17 (AdIL-17) was constructed as reported previously (9). Briefly, pACCmIL-17 vector was cotransfected into 911 cells with *Xba*I-restricted AdCMVLacZ DNA by calcium-phosphate precipitation. AdIL-17 clones were screened by PCR, and protein production was confirmed by a muIL-17 bioassay (9). All lots of recombinant adenovirus (Ad) contained less than 1 endotoxin U/ml as measured by the *Limulus* amebocyte lysate assay (BioWhittaker, Walkersville, MD). The recombinant replication-deficient adenovirus AdCMVLuc (AdControl) was used as a control vector throughout the study. All virus preparations had a PFU-particle ratio of <100:1 (9). A total of 10^7 PFU of IL-17 virus and control virus represents 6.8×10^8 and 8.9×10^8 viral particles, respectively.

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 CFA (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 dissolved in PBS, and normally first signs of arthritis onset occurs around day 25–28, in particular in the ankles.

Study protocol

CIA was induced in male DBA-1 mice as described above. Just before expected onset of CIA (day 25), mice were scored visually for the appearance of arthritis. Mice without macroscopic signs of arthritis in the paws were selected. To investigate the endogenous role of IL-17, mice were i.p. injected at alternated days (total of four injections) from day 25, with different doses of muIL-17R:Fc or BSA. Mice were macroscopically scored for arthritis incidence and severity at alternated days. X-ray analysis was performed at the end of the experiment. To examine potential amplifying activity of IL-17, DBA-1 mice were immunized with a lower dose of bovine CII (50 μg of collagen), as described above. Just before expected onset of CIA (day 25), 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 10^7 PFU per 6 μl of either an IL-17 expressing (AdIL-17) or a control vector (AdControl). Previously, we showed that this dose of adenoviral vector did not induce any inflammatory response after i.a. injection in the mouse knee joint (25). Five or 10 days after the 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 and severity score was recorded as described previously (26). Thereafter, knee joints were isolated and processed for light microscopy.

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 performed by two independent observers without knowledge of the experimental groups.

Radiology

At the end of the experiment, ankle and knee joints were isolated and used for x-ray analysis as a marker for joint destruction. X-ray photographs were carefully examined with a stereo microscope, and joint destruction was scored on a scale from 0 to 5, ranging from no damage to complete destruction of the joint (27).

Determination of IL-17, TNF-α, IL-1α, and IL-1β protein

To determine the levels of TNF-α, IL-1α, and IL-1β in washouts of joint tissue, patellae with adjacent synovium were isolated in a standardized manner from knee joints as described previously (13). Patella with adjacent synovium was 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 room temperature. After supernatant was harvested, the cytokine levels of muIL-1α, muIL-1β, and muTNF-α were measured by RIA (28). The sensitivity of the RIA IL-1α, IL-1β, and TNF-α is 20, 20, and 40 pg/ml, respectively. The cytokine levels of muIL-17 was 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). Nonspecific binding sites were blocked by 1 h of incubation at 37°C with 1% BSA in PBS-Tween. The supernatants from the patella cultures were tested by 3 h of incubation at 37°C. The plates then were 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 H_2O_2. Absorbance was measured at 492 nm with an ELISA plate reader (Titertek Multiscan MCC/340; Labsystems, Helsinki, Finland). The cytokine concentration in the samples was calculated as pg/ml with recombinant muIL-17 as a standard. The sensitivity of the IL-17 ELISA is 50 pg/ml. To measure muIL-17 levels <50 pg/ml, a commercial muIL-17 ELISA kit was used (Quantikine M; R&D Systems). The sensitivity of this ELISA is <5 pg/ml.

Determination of anti-collagen Abs

IgG1 and IgG2a anti-collagen Abs titers against bovine CII were determined by an ELISA. Briefly, plates were coated with 10 μg of bovine CII, and thereafter nonspecific binding sites were blocked with 0.1 M ethanolamine (Sigma, St. Louis, MO). Serial 1:2 dilutions of the sera were added, followed by incubation with isotype-specific goat anti-mouse peroxidase (Southern Biotechnology Associates, Birmingham, AL) and substrate (5-aminosalicylic acid; Sigma). The numbers of serum samples that were analyzed varies from four to six samples per group per time point in both the endogenous IL-17 blocking experiments as well as systemic IL-17 overexpressing conditions and their control groups. Plates were read at 492 nm. Titers were expressed as the mean $\pm$ SD dilution that gave the half-maximal value.

Isolation of RNA

Mice were sacrificed by cervical dislocation, and the patella and adjacent synovium were immediately dissected (29). Synovium biopsy tissue was

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taken from six patella specimens. Two biopsy specimens with a diameter of 3 mm were punched out with a biopsy punch (Stifle, Wachtersbach, Germany): one from the lateral side and one from the medial side. Three lateral and three medial biopsy samples were pooled to yield four samples per group of six mice. The synovium samples were immediately frozen in liquid nitrogen. Synovium biopsy samples were ground to powder with a microdismembrator II (B. Braun, Melsungen, Germany). Total RNA was extracted in 1 ml of TRIzol reagent (Life Technologies), a monophasic solution of phenol and guanidine isothiocyanate, which is an improved single-step RNA isolation method based on the method described by Chomczynski and Sacchi (30).

PCR amplification

One microgram of synovial RNA was used for RT-PCR. Messenger RNA was reverse-transcribed to cDNA with 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 *Tag* polymerase (Life Technologies) in standard PCR buffer (20 mM Tris-HCl, pH 8.4, and 50 mM KCl; Life Technologies). The mixture was overlaid with mineral oil and amplified in a thermocycler (Omnigene; Hybaid, Ashford, U.K.). cDNA was amplified for 40 cycles. Message for GAPDH was amplified at 55°C with the primers described elsewhere (27). Sequence of gene-specific primers IL-17 (accession no. U43088) for RT-PCR was: 5′-mIL-17, TCT CAT CCA GCA AGA GAT CC; 3′-mIL-17, AGT TTG GGA CCC CTT TAC AC. Message for IL-17 was amplified at 60°C.

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 or Safranin O. Histopathological changes were scored with 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 (exudate) and synovial tissue (infiltrate). Proteoglycan depletion was determined with 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 and bone. Cartilage 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. Bone destruction was graded on a scale of 0–5, ranging from no damage to complete loss of the bone structure. Histopathological changes in the knee joints were scored in the patella and femur/tibia regions on five semiserial sections of the joint spaced 70 μm apart. Two observers without knowledge of the experimental group, as described earlier (27), performed scoring.

Streptococcal cell wall (SCW) preparation and induction of SCW arthritis

Streptococcus pyogenes T12 organisms were cultured overnight in Todd-Hewitt broth. Cell walls were prepared as described previously (32). The resulting 10,000 $\times$ *g* supernatant was used throughout the experiments. These preparations contained 11% muramic acid. Genetic background of IL-1β-deficient mice prevents induction of CIA in these mice. Therefore, unilateral arthritis was induced in normal and IL-1β-deficient mice by i.a. injection of 25 μg of SCW (Rhamnose content) in 6 μl of PBS into the right knee joint of naive mice. As a control, PBS was injected into the left knee joint. To investigate the effects of local IL-17 overexpression in this model, 10^7 PFU of AdIL-17 or the control vector were i.a. injected in the knee joint 18 h before inducing SCW arthritis. Four and 10 days after SCW arthritis induction, the effects on inflammation were analyzed.

Statistical analysis

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

Results

IL-17 expression in CIA

To investigate IL-17 expression during early onset of CIA, DBA mice were immunized with CII. Low levels of systemic IL-17 protein were found over a period of 10 days after CII booster injection (range, 6–13 pg/ml). Around expected onset, synovium samples were taken at days 26, 28, 30, and 32. Histologic analysis showed negligible and mild inflammation at days 26 and 28 (0.1 $\pm$ 0.04 and 0.5 $\pm$ 0.4, respectively), which was gradually increasing during time (day 30, 1.8 $\pm$ 1.2 and day 32, 2.2 $\pm$ 1.5). IL-17 expression was examined with RT-PCR. IL-17 mRNA expression was noted at day 26 and seems fully expressed at days 28, 30, and 32 (Fig. 1).

Blocking endogenous IL-17 suppresses the onset of CIA

To examine the role of IL-17, endogenous IL-17 was blocked at expected onset of CIA. To this end, CII-immunized DBA-1 mice were given a booster injection on day 21. At days 25, 27, 29, and 31, mice were treated with different concentrations of soluble muIL-17 receptor protein (muIL-17R:Fc). Blocking endogenous IL-17 dose-dependently suppresses the arthritis incidence (Fig. 2*A*). At day 33, significant inhibition of the clinical score was noted in mice treated with 25, 75, and 150 μg of muIL-17R:Fc, although no difference in clinical score was found between the 75- and 150-μg treated groups at this time point.

In a second and third experiment, CII immunized DBA-1 mice were treated with 75 μg of muIL-17R:Fc at days 25, 27, 29, and 31. At day 31, significant difference was noted in the number of animals with arthritis (100% control vs 69% muIL-17R:Fc). In addition, at days 31 and 33, severity of arthritis was significantly suppressed after muIL-17R:Fc treatment ($p = 0.04$ and $p = 0.01$, respectively; Fig. 2*B*). Apart from the analysis of clinical inflammation, ankle joints were taken at the end of the experiment, and joint damage was analyzed by x-ray. Interestingly, radiographic analysis showed less joint damage in the muIL-17R:Fc-treated group compared with the control group ($p = 0.02$; Fig. 2*C*), indicating a role of endogenous IL-17 not only in inflammation but also in joint destruction.

Blocking endogenous IL-17 may influence the development of arthritis immunity. Therefore, we examined whether IL-17 plays a role in IgG1 and IgG2a anti-collagen Ab production. IgG1 and IgG2a were determined in sera at day 33. No difference in IgG1 (control, 781 $\pm$ 404 vs sIL-17R:Fc, 432 $\pm$ 255) and IgG2a (control, 714 $\pm$ 247 vs sIL-17R:Fc, 684 $\pm$ 358) production was found after blocking endogenous IL-17. Apart from measuring IgG1 and IgG2a anti-collagen Ab levels, we also analyzed the effects of blocking IL-17 in T cell responses to collagen. No differences in T cell responses to murine collagen peptides were noted between the sIL-17RFc-treated group and the control group (data not shown).

Systemic IL-17 gene transfer

To investigate the potential of IL-17 to enhance arthritis, gene transfer with an adenoviral vector expressing IL-17 was envisaged. CII-immunized mice were i.v. injected with 1 $\times$ 10^8 PFU of AdIL-17 or AdControl 20 days after immunization. On day 21, mice were given a booster injection with CII. At day 23, 70% arthritis incidence was already noted in mice injected with

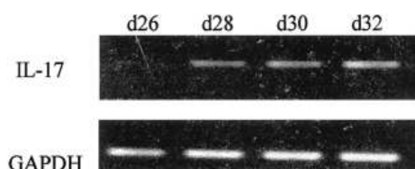

FIGURE 1. Local IL-17 expression in synovium of mice with CIA. DBA-1 mice were immunized with CII, and a booster injection was given at day 21. Synovium samples were taken at days 26, 28, 30, and 32. IL-17 expression was examined by RT-PCR, as described in *Materials and Methods*.

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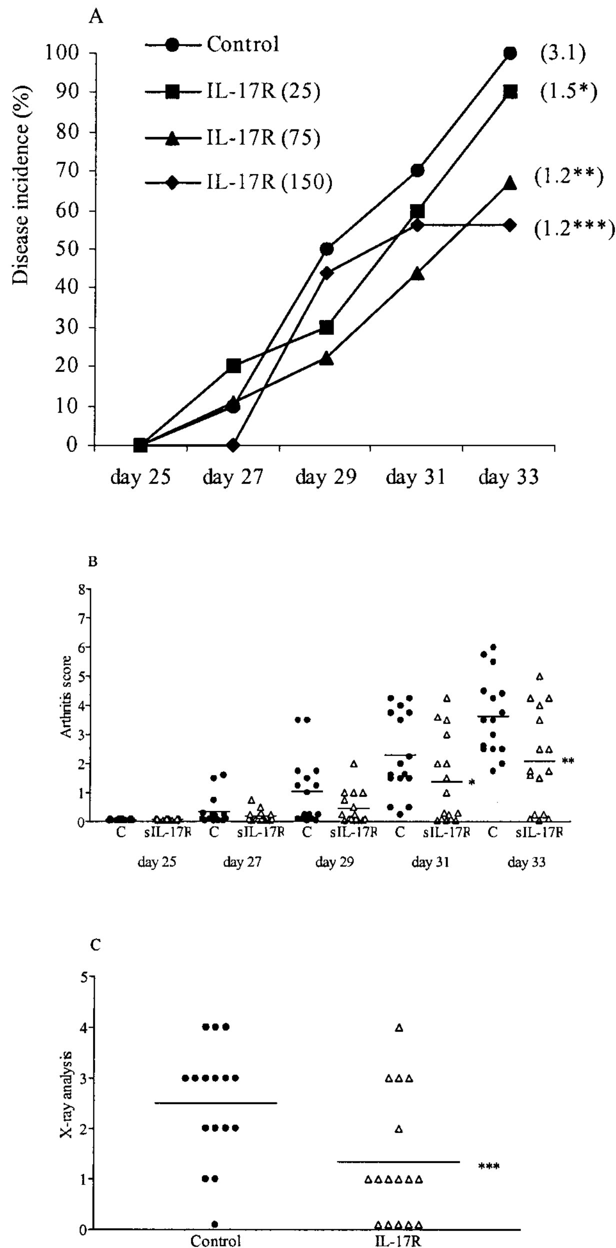

FIGURE 2. Effects of blocking endogenous IL-17 during onset of CIA. DBA-1 mice were immunized with CII, and a booster injection was given at day 21. *A*, Just before expected onset (day 25), mice were treated with 25, 75, or 150 μg of muIL-17R: Fc (i.p.) at alternated days. The appearance of arthritis was assessed, and arthritis was scored for severity (between brackets, day 33: Control, 3.1 ± 0.6; IL-17R (25), 1.5 ± 0.4; IL-17R (75), 1.2 ± 0.5; IL-17R (150), 1.2 ± 0.5). Results are the mean ± SEM of at least nine mice per group. *, $p = 0.04$. **, $p = 0.02$; ***, $p = 0.03$, compared with control group, by Mann-Whitney rank sum test. *B* and *C*, Mice were i.p. injected with 75 μg of muIL-17R: Fc at alternated days, started just before onset (day 25). The appearance of arthritis was visually scored for severity (arthritis score; *B*) and ankles joints were analyzed for joint damage by x-ray (*C*). Results are the mean ± SD of two separate experiments with a total of 16 mice per group. *, $p = 0.04$. **, $p = 0.01$. ***, $p = 0.02$ vs control group, by Mann-Whitney rank sum test.

AdIL-17 compared with 10% in the control vector group (Fig. 3*A*). The severity of arthritis was significantly enhanced after systemic IL-17 gene transfer (Fig. 3*B*), indicating that IL-17 accelerates CIA expression.

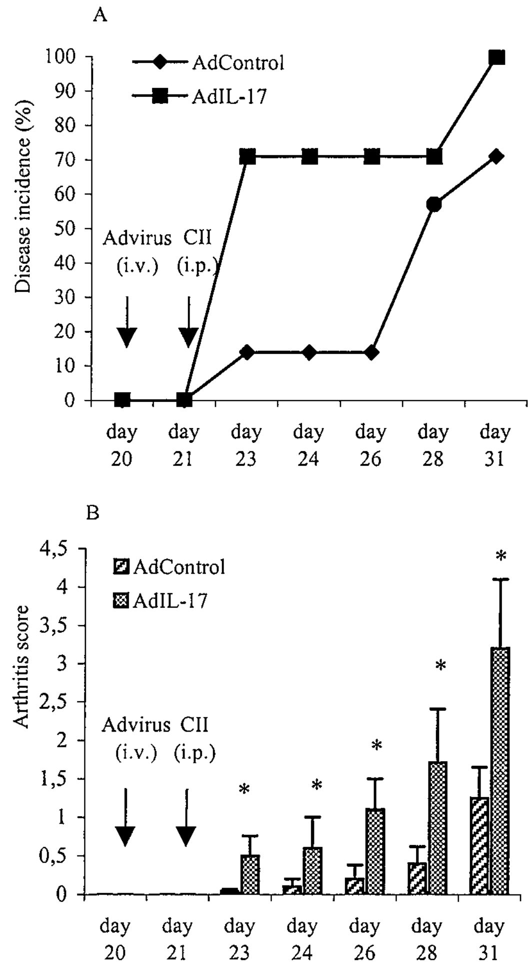

FIGURE 3. Systemic IL-17 gene transfer accelerates onset of CIA. CII-immunized DBA-1 mice were i.v. injected with 10^8 PFU of AdIL-17 or AdControl 1 day before booster injection. The appearance of arthritis was assessed (*A*) and arthritis was scored for severity (*B*). Results are the mean ± SD of at least 14 mice per group. *, $p < 0.05$ vs control group, by Mann-Whitney Rank sum test.

Low levels of IL-17 were measured in sera over a period of 10 days after the CII booster injection. Injections (i.v.) of AdIL-17 markedly increased systemic IL-17 expression during this time period, with a maximum at 1 day after the viral injection (day 21, 94 ± 12 pg/ml to day 31, 34 ± 2 pg/ml). This is a factor 8.5 and 2.5 higher compared with the control vector group at day 21 and day 31, respectively.

At days 24, 29, and 31, no differences were found in IgG1 and IgG2a anti-collagen Ab levels in serum between the AdIL-17 and the control vector group, although an increase in both IgG1 and IgG2a anti-collagen Ab levels was observed in both groups after CII booster injection (data not shown).

Local IL-17 gene transfer

Because systemic IL-17 gene transfer enhanced CIA and no direct influences of IL-17 was noted in Ig production, we examined whether local IL-17 had amplifying activity when injected in the knee joint of CII-immunized mice. To start with, naive mice were i.a. injected in the right knee joint with 1 × 10^7 PFU of AdIL-17 or AdControl, and IL-17 levels were measured at different time points in washouts of joint tissue. High levels were found in the first 7 days after a single injection of AdIL-17 with a maximum at

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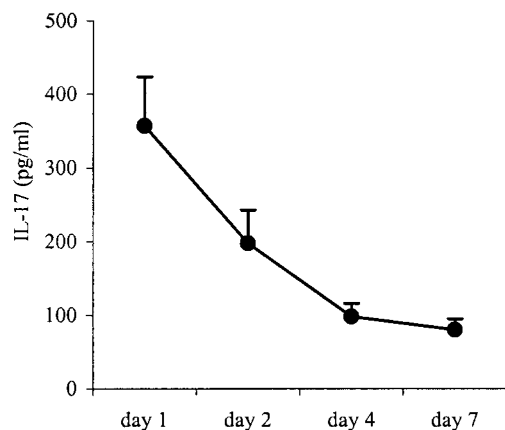

FIGURE 4. Adenoviral vector-mediated IL-17 expression in the knee joint of naive mice. A total of 10^7 PFU of AdIL-17 was i.a. injected in the right knee joint of naive mice. At days 1, 2, 4, and 7, patellae with adjacent synovium were isolated in a standardized manner from knee joints and cultured for 1 h in 200 μl of RPMI 1640 medium at room temperature; thereafter, the culture supernatants were assayed for IL-17 by ELISA. The same dose of the control vector i.a. injected in the left knee gave rise to undetectable levels of IL-17 (not graphed). Results are the mean ± SD of five mice. The detection limit of the IL-17 ELISA is <5 pg/ml.

day 1 (Fig. 4). No detectable levels of IL-17 were found in washouts from the contralateral knee or the AdControl vector-injected knee (data not shown).

Influx of polymorphonuclear cells (PMNs) after local IL-17 overexpression in naive DBA-1 mice

Histologic analysis of naive mouse knee joints injected with 1×10^7 PFU of AdIL-17 revealed joint inflammation at days 2, 5, and 10, which gradually increased during time (Table I). Predominantly, influx of PMNs was noted at day 2, and at days 5 and 10 mononuclear cells also were found. No chondrocyte death and cartilage erosion were observed during this time period, and only mild bone erosion in the femur region at day 10 was found. However, local IL-17 expression induces significant proteoglycan depletion (Table I). Although marked influx of PMNs was apparent, these cells did not stick to the cartilage layer (data not shown).

Local IL-17 overexpression aggravates synovial inflammation

DBA-1 mice were immunized with suboptimal concentration of CII, and shortly before expected onset of CIA, a single injection of different doses of 10^5, 10^6, and 10^7 PFU of either AdIL-17 or AdControl were i.a. injected in the right or left knee joint, respectively. Five days after the viral injection, knee joints were analyzed and severity of arthritis was scored. Local IL-17 overexpression during the early phase of CIA dose-dependently enhanced the onset and promoted synovial inflammation compared with mice injected with control vector (Table II).

In the next set of experiments, a single injection of 1×10^7 PFU of AdIL-17 or control vector was given in the right knee joint. Five days after i.a. injection of AdIL-17, a 100% incidence was already noted in the right knee joints of the AdIL-17 group. In contrast, 36 and 56% incidence was observed in joints injected with the control vector at days 5 and 10, respectively (Fig. 5).

Apart from the incidence, also the severity of the arthritis was markedly enhanced ($p < 0.001$; Fig. 5). Furthermore, histologic analysis showed significantly more inflammatory cells in the synovium (infiltrate; $p < 0.001$) and joint space (exudate; $p < 0.001$) compared with the control vector group (Fig. 6*A*). In addition, we have analyzed in these experiments the effects on the ipsilateral paw. Local IL-17 gene transfer in the knee joint significantly increased the arthritis severity in the ipsilateral paw (Fig. 6*B*).

IL-17 enhances joint destruction

In addition to inflammatory aspects, 5 days after i.a. injection of the adenoviral vectors, chondrocyte death was observed in the control vector group. However, local overexpression of IL-17 markedly enhanced the degree of chondrocyte death ($p < 0.001$; Figs. 6*A* and 7). PMNs were heavily sticking to the cartilage of the patella and femur region after IL-17 overexpression at day 5, a phenomenon hardly seen in the control group. Scant cartilage surface erosions was noticed in the control vector group at day 5, whereas pronounced cartilage destruction was found in the IL-17 overexpression group (AdControl, 0.01 ± 0.1 vs AdIL-17, 0.6 ± 0.2; $p < 0.001$; Fig. 7).

Radiographic analysis revealed enhanced knee joint erosion at day 5 ($p = 0.01$) and joint destruction at day 10 ($p < 0.001$) in the IL-17 group compared with the control group (Figs. 8 and 9). Histologic analysis showed a mild degree of bone erosion in the control group at day 5. However, pronounced bone erosion was then already noted in the cortical bone of the patella (AdControl, 0.3 ± 0.6 vs AdIL-17, 1.8 ± 0.5; $p < 0.001$) and femur/tibia region (AdControl, 0.1 ± 0.2 vs AdIL-17, 0.7 ± 0.2; $p < 0.001$) in the IL-17 group.

Elevated levels of IL-1β in the synovium by local IL-17

In vitro studies have shown that IL-17 can stimulate the production of IL-1β and TNF-α (5, 12). Furthermore, we have shown previously the role of IL-1 in cartilage destruction in CIA (17, 33). Therefore, we investigated the effects of local IL-17 overexpression on the production of the proinflammatory cytokines IL-1 and TNF-α in the synovium. As shown in Table III, local IL-17 enhances the protein expression of IL-1β ($p = 0.009$). No elevated levels of TNF-α or IL-1α were induced by local IL-17 (Table III). This suggests that the local IL-17 effects can be mediated by IL-1β production in the synovium.

Table I. *Histologic analysis of the impact of local IL-17 overexpression in the knee joint of naive mice*[a]

	Inflammation[b]		Cartilage Damage[b]		
Day	Infiltrate	Exudate	PG depletion	Chondrocyte death	Surface erosion
2	1.0 ± 0.4	0.9 ± 0.5	0.9 ± 0.3	0.04 ± 0.1	0
5	1.6 ± 0.8	0.6 ± 0.6	1.7 ± 0.6	0.08 ± 0.1	0
10	2.4 ± 0.4	1.3 ± 0.6	2.3 ± 0.2	0.04 ± 0.1	0

[a] Naive DBA-1 were i.a. injected with 10^7 PFU of AdIL-17 or the control vector. At days 2, 5, and 10 knee joints were taken for histology.
[b] Synovial inflammation was score based on the amount of cells in the synovium (infiltrate) and joint space (exudate). Inflammation and cartilage damage were scored on a scale of 0–3. Results are the mean ± SD of at least six mice per group per time point. No signs of inflammation and joint damage were found after i.a. injection of the same dose of the control vector. PG, Proteoglycan.

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Table II. *Dose response study of AdIL-17 in the knee joint*[a]

	Dose (PFU)	Arthritis Score (day 5)
AdControl	10^5	1.3 ± 0.4
AdIL-17	10^5	1.2 ± 0.5
AdControl	10^6	0.5 ± 0.4
AdIL-17	10^6	1.7 ± 0.3*
AdControl	10^7	0.4 ± 0.7
AdIL-17	10^7	2.0 ± 0.1*

[a] Just before expected onset (day 25) CII-immunized mice were i.a. injected with different doses of either AdIL-17 or AdControl in the right and left knee joint, respectively. Five days after the viral injection, knee joints were analyzed, and severity of arthritis was scored on a scale of 0–2. Results are the mean ± SD of at least eight mice per group. *, $p < 0.001$ vs control group, by Mann-Whitney rank sum test.

Local IL-17 promotion of CIA is IL-1αβ-independent

To investigate the contribution of the proinflammatory cytokine IL-1 on the IL-17-induced inflammation, we used specific Abs to block IL-1αβ activity. Remarkably, blocking of IL-1 had no effect on IL-17-induced acceleration of arthritis in the knee ($p < 0.001$; Table IV). Moreover, x-ray analysis at day 5 revealed no difference in IL-17-induced joint damage between the anti-IL-1 group and the control group (1.0 ± 0.3 SEM and 1.0 ± 0.3 SEM, respectively), although significant difference in joint damage was found between the AdIL-17 and the control vector group at this time point (1.0 ± 0.3 SEM and 0.1 ± 0.002 SEM, respectively; $p = 0.02$). This implies that the IL-17-induced inflammation and joint damage are independent of IL-1.

IL-17 exaggerates SCW arthritis, independent of IL-1

We further examined the IL-1-independent potential of IL-17 to enhance joint pathology in SCW arthritis. This model was induced in normal and IL-1β-deficient mice. Interestingly, local overexpression of IL-17 enhanced chronicity and erosive character of the SCW arthritis in the control mice ($p = 0.008$). Of high interest, similar exaggeration of arthritis was found in the IL-1$\beta^{-/-}$ mice compared with the control mice (Fig. 10). This strongly underscores an IL-1-independent role of IL-17.

Discussion

This study clearly demonstrated contribution of IL-17 in joint inflammation as well as tissue destruction in an autoimmune model of arthritis. In addition, it identifies an IL-1 independent role of IL-17 in the pathogenesis of arthritis.

IL-1 and TNF-α are key mediators in the pathology of arthritis, driving enhanced production of cytokines, chemokines, and degradative enzymes (34). Neutralizing TNF-α or IL-1β yielded promising results in controlling chronic inflammation and cartilage degradation, respectively. However, none of these treatments cured the disease. Therefore, it is tempting to speculate that cytokines or factors other than IL-1 and TNF-α also participate in the proinflammatory cytokine cascade. IL-17 has been found to stimulate the production of IL-1 and TNF-α from macrophages (5) and triggers human synoviocytes to produce IL-6, IL-8, GM-CSF, and PGE_2 (1, 12), suggesting that IL-17 could be an upstream mediator in the pathogenesis of arthritis. In the present study, we found amelioration of CIA after blocking of endogenous IL-17. Furthermore, IL-17 overexpression during onset aggravates synovial inflammation and joint destruction, a process that was independent

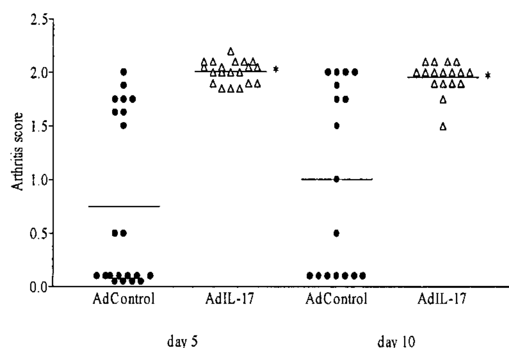

FIGURE 5. Effects of AdmIL-17 in the mouse knee joint with CIA. CII-immunized DBA-1 mice were i.a. injected in the right knee joint with 1.10^7 PFU of either AdIL-17 or AdControl before onset of CIA was noted. At days 5 and 10 after i.a. injection of the viral vector, mice were sacrificed by cervical dislocation, and the skin of the knee joint was removed. The appearance of arthritis in the injected joints was visually scored for severity (arthritis score). Results are the mean ± SD of two separate experiments with a total of at least 17 mice per group.

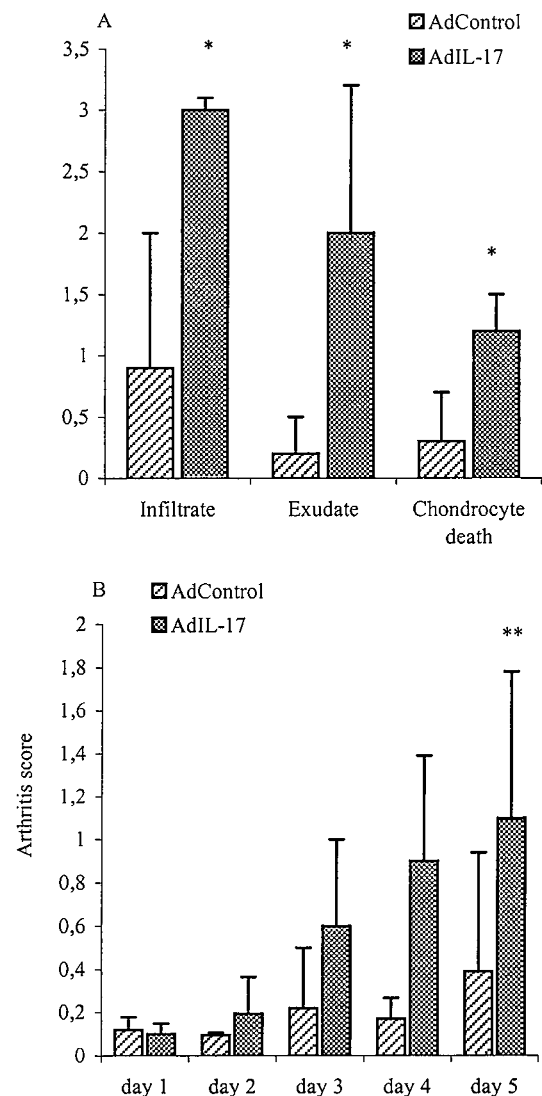

FIGURE 6. Analysis of the inflammatory aspects of local IL-17 overexpression in the knee joint of mice with CIA. *A*, At day 5, knee joints were taken for histology. Synovial infiltrate, exudate, and chondrocyte death were scored on a scale of 0–3. *B*, Appearance of arthritis in the ipsilateral paws was visually scored for severity (arthritis score). Results are the mean ± SD of two separate experiments with at least eight mice per group per experiment. *, $p < 0.001$; **, $p < 0.04$ vs control vector group, by Mann-Whitney rank sum test. For details, see Fig. 5.

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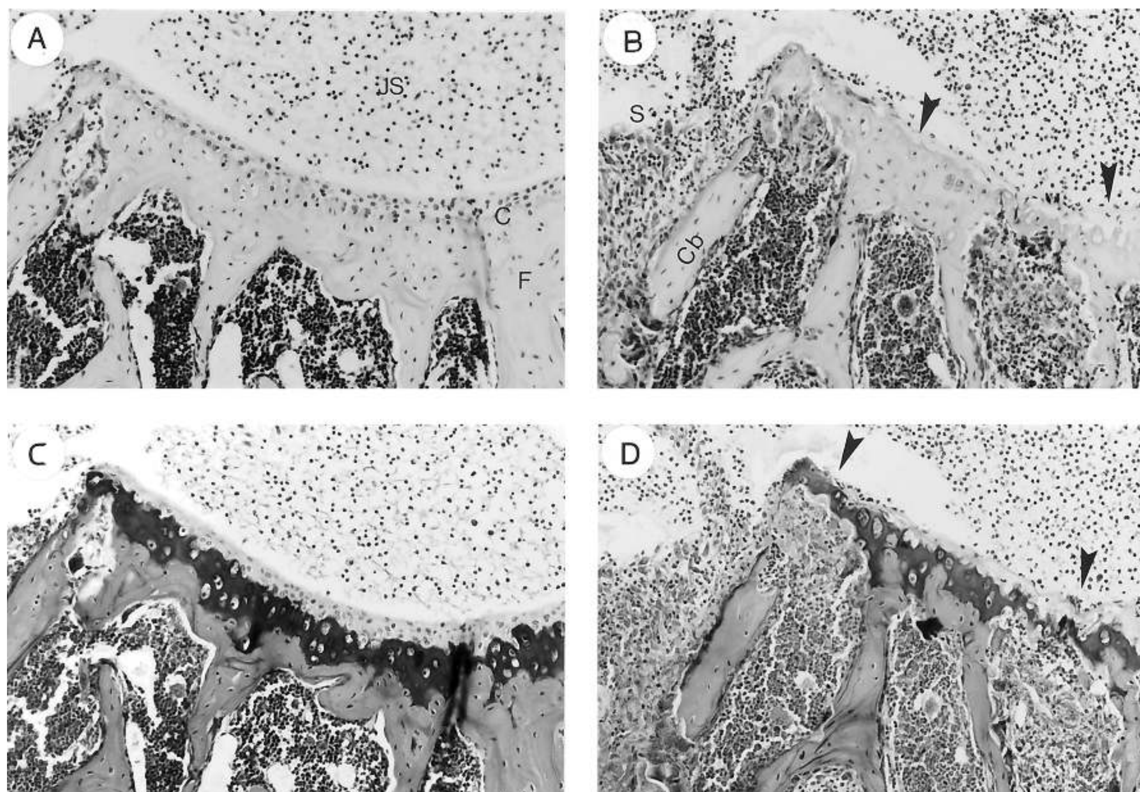

FIGURE 7. Effects of local IL-17 expression on joint pathology. *A* and *C,* Arthritis knee joint of mice 5 days after i.a. injection of 10^7 PFU of AdControl vector. *B* and *D*, Knee joint of a mouse 5 days after i.a. injection of 10^7 PFU of AdIL-17. Note the pronounced chondrocyte death and cartilage surface erosion (arrows). *A–D*, Original magnification, ×200. Hematoxylin and eosin was used in *A* and *B*, and safranin O staining was used in *C* and *D*. C, Cartilage; F, femur; JS, joint space; S, synovium; Cb, cortical bone.

of IL-1. Therefore, our data put IL-17 on the list of important proinflammatory cytokines in experimental arthritis with destructive capacity, independent of IL-1.

RA is considered a Th1-associated disease (35). However, the factors that initiate and sustain Th1 responses in RA synovium still are not identified. IL-15 may replace the requirement for IL-2, as it has been shown that it has biological functions similar to those of IL-2. IL-15 can recruit and activate $CD45RO^{+}$ memory T cell subset into the synovial membrane and can induce TNF-α production in RA (36, 37). Interestingly, these T cell subsets are IL-17 producer cells after stimulation, and it has been shown that IL-15 triggers IL-17 production in vitro (22). From these studies, it is not clear whether IL-17 operates downstream of IL-15 and whether IL-17 has a direct role in T cell activation. Although direct T cell activation by IL-17 in vivo has not been shown, a role for IL-17 in allogenic T cell proliferation has been suggested (7). IL-17 can promote the development of dendritic cell progenitors by increased surface expression of CD11c, CD40, CD80, CD86, and MHC class II Ags. IL-17 did not significantly affect the phenotype or function of mature dendritic cells. In the present study, no differences in T cell responses to collagen and in IgG1 or IgG2a anti-collagen Ab levels were found compared with the control group after systemic

FIGURE 8. Local IL-17 expression enhances knee joint erosion during CIA. CII-immunized mice with a booster injection on day 21 were i.a. injected in the right knee joint on day 25 with 10^7 PFU of AdIL-17 and in the left knee joint with the same dose of AdControl before onset of CIA was noted (day 25). Ten days after the viral injection, knee joints were analyzed for joint damage by x-ray. Results are the mean ± SD of nine mice per group. *, $p < 0.001$ vs control group, by Mann-Whitney rank sum test.

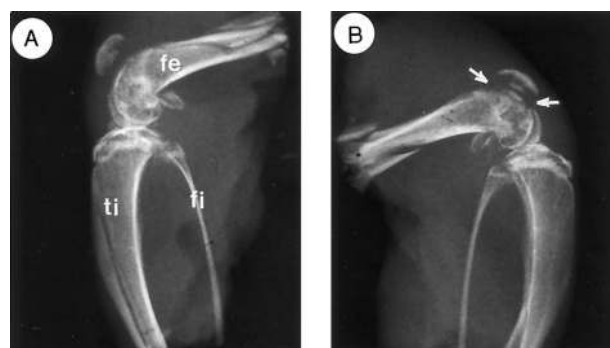

FIGURE 9. Effects of AdIL-17 on joint damage and bone structure in CIA. Arthritic knee joint of a mouse 10 days after i.a. injection of 10^7 PFU of AdControl (*A*) or AdIL-17 (*B*). Note the enhanced joint destruction in the IL-17 group (arrows). Fe, Femur; fi, fibula; ti, tibia. For details, see Fig. 8.

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Table III. *Effect of local IL-17 on proinflammatory cytokine expression in synovium during CIA*[a]

	Dose (PFU)	IL-1α[b] (pg/ml)	IL-1β[b] (pg/ml)	TNFα[b] (pg/ml)
AdControl	10^7	<40	57 ± 99	<40
AdIL-17	10^7	<40	433 ± 121*	<40

[a] CII-immunized DBA-1 mice were suboptimal immunized with type II collagen and on day 21 a booster injection was given. Just before expected onset, mice were i.a. injected with 10^7 PFU of AdIL-17 or AdControl. Five days after the i.a. injection of the viral vector, patella washouts were taken and assayed for IL-1 and TNF-α levels. Note the marked increase of IL-1β in the AdIL-17 group.

[b] Patella with adjacent synovium were isolated in a standardized manner from the knee joints and cultered for 1 h in 200 μl RPMI 1640 medium at room temperature; thereafter, IL-1α, IL-1β, and TNF-α levels were measured in these culture supernatants using an IL-1α, IL-1β, and TNF-α specific radioimmunoassay. Results are the mean ± SD of at least six patella washouts per group.

*, $p = 0.009$ vs control vector group, by Mann-Whitney rank sum test.

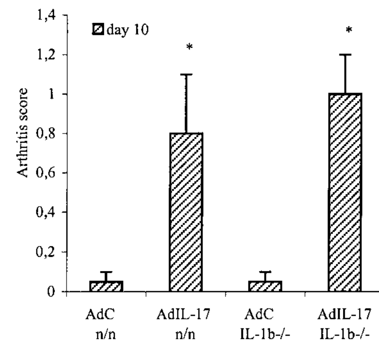

FIGURE 10. Effect of local IL-17 in SCW arthritis. Normal and IL-1β-deficient mice were i.a. injected with 10^7 PFU of AdIL-17 or AdControl. After 18 h, SCW arthritis was induced by i.a. injection of 25 μg of SCW fragments. Ten days later, arthritis was visually scored for severity (arthritis score). Results are the mean ± SD of at least six mice per group. *, $p = 0.008$ vs control group, by Mann-Whitney rank sum test.

overexpression of IL-17 around booster injection or blocking endogenous IL-17 around the onset of CIA. This implies that IL-17 did not have a role in the development of immunity against CII.

IL-17 appears a novel target in T cell-mediated inflammatory disease, playing a role upstream in the pathologic process. In the present study, we found that blocking endogenous IL-17 results in suppression of CIA, including reduction of joint damage. Apart from direct blocking of endogenous IL-17 with soluble IL-17 receptor, previously we showed that overexpression of IL-4 down-regulated IL-17 and IL-1 expression and prevented cartilage and bone erosion (26, 27). In contrast, the promotion of CIA in the knee joint by local IL-17 overexpression was not restricted to the knee joint alone. Local overexpression of IL-17 in the knee joint also accelerates the arthritis incidence in the ipsilateral paw. As IL-17 can increase IL-1 and TNF-α production in macrophages, blocking IL-17 will also decrease IL-1 and TNF-α actions. These findings imply that regulation of the production and activity of IL-17 may have important consequences in the net production of proinflammatory cytokines in the synovium and in tissue-destructive processes.

Apart from its role in the inflammatory process, IL-17 may be an important catabolic mediator in tissue destruction. Involvement of IL-17 in cartilage disturbance has been shown in vitro (13), and it contributes to joint degradation in RA (38). Furthermore, we showed that i.a. injections of adenoviral IL-17 in the knee joint of naive mice results in proteoglycan depletion, which is in line with the observation reported by Dudler et al. (39). Although these studies indicate catabolic effects of IL-17, the role of IL-1, which is the most potent catabolic mediator in the arthritic process, cannot be excluded. Low levels of IL-17 and IL-1 have additive or synergistic effect on joint pathology in vitro (38). In the present study, we showed that local overexpression of IL-17 in the knee joint of CII-immunized mice resulted in elevated levels of IL-1β in the synovium. No increase in TNF-α and IL-1α levels was found. However, this study did not rule out the possibility that TNF-α and IL-1α were elevated at earlier time points. It has been shown that TNF-α and IL-1α play a role especially in early CIA. IL-1β plays a dominant role in early and late CIA. The fact that IL-17 still induced enhancement of arthritis in the absence of IL-1 indicates an IL-1 independent role of IL-17. Furthermore, these data suggest that IL-17 not only play a promotive role upstream in the T cell-mediated inflammatory process but can also function as an independent catabolic mediator. Taking this together, IL-17 seems to be a strong IL-1-inducing cytokine in vivo and can alone or together with IL-1 play a key role in destructive arthritis.

In the present study, we found that local overexpression of IL-17 in the knee joint leads to enhanced influx of PMNs. This is in line with earlier reports showing that IL-17 stimulates granulopoiesis in mice (8, 9). The main difference between IL-17 overexpression in naive and CII-immunized DBA-1 mice was the observation that in CIA, PMNs were heavily sticking to patella and femur cartilage, a phenomenon that was not observed in naive mice. Interestingly, under both conditions, local IL-17 induced proteoglycan depletion. However, no chondrocyte death and cartilage erosion was observed in the naive mice after local IL-17 overexpression. In contrast, local IL-17 aggravates cartilage erosion in CIA. It has been shown that increased elastase activity was associated with neutrophil recruitment by IL-17 in airways in vivo (40). The elevated neutrophil influx and subsequent sticking to anti-CII immune complexes in the cartilage surface layer probably releases oxygen species and proteolytic enzymes present in the PMNs directly into the surface of the cartilage, thereby escaping inhibitors present in the synovial fluid (41, 42). PMNs may play an active role in cartilage destruction and need this close contact to cartilage to accomplish cartilage damage.

Here, we report that IL-17 accelerates bone erosion in CII-immunized mice and blocking endogenous IL-17 suppresses joint damage. Activated T cells may play a regulatory role in bone loss and joint destruction through RANKL in vivo (43), and it has been shown that T cell IL-17 may be a crucial cytokine for osteoclastic bone resorption in vitro via RANKL expression (15, 44). Osteoclasts are potent bone-resorbing cells, and RANKL has been shown to be a key regulator of osteoclastogenesis. Moreover, RANKL binds to its receptor, RANK, and the RANKL/RANK balance seems of crucial importance in osteoclastogenesis and the

Table IV. *Effects of anti-IL-1 treatment on the IL-17-induced synovial inflammation*[a]

	Dose (PFU)	Arthritis Score		Suppression After IL-1 Treatment (%)
		Control	Anti-IL-1	
AdControl	10^7	0.5 ± 0.7	0.1 ± 0.1	81
AdIL-17	10^7	2.0 ± 0.1	2.0 ± 0.1	0

[a] CII-immunized mice were i.a. injected with 10^7 PFU of AdIL-17 in the right knee joint, and the same dose of the control vector was injected in the left knee joint. Two hours before virus injection, mice were i.p. injected with anti-IL-1 or NKS, as a control. Five days later, mice were sacrificed by cervical dislocation, and the skin of the knee joint was removed. The appearance of arthritis in the injected joints was visually scored for severity (arthritis score) on a scale of 0–2. Results are the mean ± SD of at least eight mice per group.

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bone erosion process. We found RANKL and RANK protein expression in the synovium and at bone erosion sites in the IL-17 group by specific immunohistochemistry (E. Lubberts et al., manuscript in preparation). The role of IL-17, in relation to IL-1 and TNF in the regulation of RANKL and osteoclastic bone resorption in vivo needs further clarification. In vitro, IL-17 had no effect on basal and IL-1β stimulated osteoclastic bone resorption, but when given together with TNF-α it increased bone resorption (44). Furthermore, TNF-α can stimulate osteoclast differentiation independent of RANKL-RANK interaction in vitro (45). Interestingly, we found that anti-IL-1 treatment had no effect on RANKL expression after local adenoviral IL-17 injection (E. Lubberts et al., manuscript in preparation). Further characterization of the role of IL-17 in relation to IL-1 and TNF-α in RANKL activity is warranted.

In conclusion, this is the first report demonstrating clear contribution of IL-17 to the pathogenesis of CIA. IL-17 plays a role in joint inflammation as well as tissue destruction. Although local IL-17 enhanced IL-1 levels in the synovium, blocking IL-1 had no effect on IL-17-induced joint inflammation and joint destruction, identifying an IL-1-independent role of IL-17. Our data make it clear that IL-17 can amplify T cell-driven arthritis, making it more destructive. These findings imply that IL-17 is a novel target for destructive arthritis, and similar processes may underlie tissue destruction in other autoimmune disorders.

Acknowledgments

We acknowledge The Central Animal Laboratory, Faculty of Medicine, University of Nijmegen for animal care. We thank Ineke Verschueren for technical assistance.

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