Musculoskeletal Pathology

Blocking of Interleukin-17 during Reactivation of Experimental Arthritis Prevents Joint Inflammation and Bone Erosion by Decreasing RANKL and Interleukin-1

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Rheumatoid arthritis is characterized by an intermittent course of disease with alternate periods of remission and relapse. T cells, and in particular the T-cell cytokine interleukin-17 (IL-17), are expected to be involved in arthritic flares. Here, we report that neutralizing endogenous IL-17 during reactivation of antigen-induced arthritis prevents joint inflammation and bone erosion. Synovial IL-17 mRNA expression was clearly up-regulated during primary arthritis and was further enhanced after antigen rechallenge. Neutralization of IL-17 significantly prevented joint swelling at day 1 of flare and significantly suppressed joint inflammation and cartilage proteoglycan depletion at day 4, as assessed by histology. Blocking IL-17 also clearly reduced bone erosions. Cathepsin K, a marker of osteoclast-like activity, and synovial RANKL mRNA expression were both suppressed. The degree of bone erosions strongly correlated with the severity of joint inflammation, suggesting that anti-IL-17 treatment reduced bone erosion by suppressing joint inflammation. Interestingly, blocking IL-17 suppressed synovial expression of both IL-1 β and tumor necrosis factor- α , whereas blocking IL-1 did not affect tumor necrosis factor- α levels. These data indicate that IL-17 is an important upstream mediator in joint pathology

during flare-up of experimental arthritis. (*Am J Pathol* 2005, 167:141–149)

Rheumatoid arthritis (RA) is a systemic joint disease characterized by progressive destructive joint inflammation. Although RA is considered an autoimmune disease, the autoantigen(s) is still not identified. Interestingly, CTLA4Ig treatment in patients with RA showed promising improvement of the ACR50 and ACR70 responses.¹ This treatment blocks the interaction of CD80/86 on antigen-presenting cells with CD28 on naïve T cells. This interaction is required for optimal T-cell activation. This study supports the theory that (CD4⁺) T cells play a key role in the pathogenesis of RA.^{2–4} The contribution of T cells in the joints can be related to antigen-dependent and -independent mechanisms, such as direct cell contact, which can induce cytokine and protease production by macrophages and fibroblasts.⁵ Furthermore, RA is characterized by an intermittent course of the disease with alternate periods of remission and relapse. The cause of a flare-up reaction during RA is unclear, and both antigen-specific and antigen-nonspecific T cells may play a role.⁴

Studies in animal models for experimental arthritis have resulted in better understanding of the role of different cell types and various cytokines during arthritic flares. In antigen-induced arthritis, the capacity to develop arthritis can be transferred by lymphocytes,⁶ in particular by cell fractions enriched for T cells, and high

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numbers of T cells can be detected in the inflamed synovium after flare-up reaction.⁷ Reactivation of chronic streptococcal cell wall arthritis in Lewis rats could be prevented by removal of T lymphocytes,⁸ and successful suppression of antigen-induced arthritis (AIA)-flare in mice treated with anti-lymphocyte serum⁹ or anti-CD4 monoclonal antibodies¹⁰ also suggests an important role for T cells in this process. In addition, during reactivation of arthritis, tumor necrosis factor- α (TNF- α) is markedly produced in the joint and peaks at 6 hours after antigen rechallenge,¹¹ suggesting a role for this cytokine at the onset of exacerbations. IL-1 is also clearly present in experimental flare reactions and is shown to be an important mediator in joint inflammation and cartilage pathology in exacerbations of murine arthritis.¹²

The discovery of the T-cell cytokine IL-17A in the synovium of RA patients raised the question of its role in (antigen-specific) flare-up reactions. IL-17A is a proinflammatory cytokine produced mainly by activated (memory) T cells. IL-17A, further referred to as IL-17, belongs to a growing family of related cytokines (IL-17A-F).^{13–16} Until now, only IL-17A has been shown to be involved in RA pathogenesis. Neutralizing IL-17 during collagen-induced arthritis (CIA) showed that IL-17 plays a role in the early and late stages of CIA.^{17,18} In addition, crossing IL-17-deficient mice with IL-1Ra-deficient mice prevented the spontaneous development of arthritis normally found in mice lacking IL-1-Receptor antagonist (IL-1Ra).¹⁹ These studies clearly show a role for IL-17 in the development of arthritis. However, the role of IL-17 in a flare-up reaction of arthritis remains to be elucidated.

In the present study, we used the AIA model because of its unique antigen-specific arthritic flare-reaction. After primary AIA, arthritis can be reactivated by a small amount of antigen, causing an antigen-specific flare-up reaction with an expected major role for memory T cells and their cytokines. AIA was provoked in C57Bl/6 mice, and flare-up was induced by a rechallenge with a small amount of antigen (methylated bovine serum albumin; mBSA) locally into the arthritic knee joint. The role of IL-17 was studied by neutralizing endogenous IL-17 with polyclonal anti-IL-17 antibodies. Here, we demonstrate the critical role of IL-17 in driving joint inflammation and cartilage depletion after antigen rechallenge. In addition, neutralizing IL-17 prevents bone erosion and results in suppressed synovial expression of proinflammatory cytokines (IL-1, TNF, and RANKL) and chemokines (KCs). These data reveal IL-17 as an important mediator in flare-up reaction, acting as an upstream mediator in destructive joint inflammation during an antigen-specific flare-up of experimental arthritis.

Materials and Methods

Animals

Male C57Bl/6 mice were obtained from Elevage-Janvier (Le Genest Saint Isle, France). Mice were housed in filter-top cages under specified pathogen-free conditions, and a standard diet and water were provided *ad*

libitum. The mice were used between 10 and 12 weeks of age. All animal procedures were approved by the institutional ethics committee.

Materials

Methylated bovine serum albumin was purchased from Sigma-Aldrich (St. Louis, MO). TRIzol Reagent, oligo-dT primers, and Moloney murine leukemia virus reverse transcriptase were obtained from Life Technologies (Breda, The Netherlands). Primers were purchased from Biolegio (Malden, The Netherlands). SYBR Green Master Mix was purchased from Applied Biosystems (ABI/PE, Foster City, CA).

Induction of Antigen-Induced Arthritis

Mice were immunized with 100 μ g of mBSA (Sigma), emulsified in 100 μ l Freund's complete adjuvant (Difco Laboratories, Detroit, MI). Injections were divided over both flanks and footpath of the forelegs. Heat-killed *Bordetella pertussis* (RIVM, Bilthoven, The Netherlands) was administered intraperitoneally as an additional adjuvant. Two subcutaneous booster injections with in total 50 μ g mBSA/Freund's complete adjuvant were given in the neck region 1 week after the initial immunization. Three weeks after these injections, primary AIA was induced by injecting 60 μ g of mBSA in 6 μ l of phosphate-buffered saline into the right knee joint, resulting in chronic arthritis. At week 3 of arthritis, 2 μ g of mBSA was injected intraarticularly into the arthritic joint to induce a flare-up of the smoldering inflammation.

Neutralizing Antibodies for IL-17 and IL-1

Polyclonal rabbit antibodies were directed against murine recombinant IL-17 (mIL-17; R&D Systems, Minneapolis, MN) or against each type of murine recombinant IL-1, and were prepared and tested as previously described.^{18,20} Flare-up of antigen-induced arthritis was induced in C57Bl/6 mice as described above. Two hours before flare-up, a single injection with the polyclonal mIL-17 or mIL-1 antibodies (200 μ l/mouse) was given intraperitoneally. As a control, the same amount of normal rabbit antibodies was injected.

RNA Isolation and RT Reaction

Mice were sacrificed by cervical dislocation, immediately followed by dissection of the patella with adjacent synovium. Two biopsies with a diameter of 3 mm were punched out using a biopsy punch (Stiefel, Wachtersbach, Germany), one from the lateral side and one from the medial side of the synovial tissue. 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 biopsy samples were ground to powder using a Microdismembrator

II (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.²¹ Thereafter, RNA was precipitated with isopropanol, washed with 70% ethanol, and re-dissolved in water. Isolated RNA was treated with DNase before being reverse transcribed into cDNA using oligo-dT primers and Moloney murine leukemia virus reverse transcriptase.

Quantitative Polymerase Chain Reaction (QPCR)

Quantitative real-time PCR was performed using the ABI/PRISM 7000 Sequence Detection System for quantification with Sybr Green and melting curve analysis (ABI/PE, Foster City, CA). Primer sequences for the reference gene GAPDH and other genes were as follows: 5'-GGC AAA TTC AAC GGC ACA-3' (GAPDH forward), 5'-GTT AGT GGG GTC TCG CTC TG-3' (GAPDH reverse), 5'-CAG GAC GCG CAA ACA TGA-3' (IL-17 forward), 5'-GCA ACA GCA TCA GAG ACA CAG AT-3' (IL-17 reverse), 5'-GGA CAG AAT ATC AAC CAA CAA GTG ATA-3' (IL-1 β forward), 5'-GTG TGC CGT CTT TCA TTA CAC AG-3' (IL-1 β reverse), 5'-CAG ACC CTC ACA CTC AGA TCA TCT-3' (TNF- α forward), 5'-CCT CCA CTT GGT GGT TTG CTA-3' (TNF- α forward), 5'-CTG AGG CCC AGC CAT TTG-3' (RANKL forward), and 5'-GTT GCT TAA CGT CAT GTT AGA GAT CTT G-3' (RANKL reverse). PCR conditions were as follows: 2 minutes at 50°C and 10 minutes at 95°C followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C, with data collection in the last 30 seconds. For all PCRs, SYBR Green Master Mix was used in the reaction. Primer concentrations were 300 nmol/L. Efficiencies (E) for all PCR primers were determined from a serial dilution series of cDNA in water: GAPDH, E = 2.09; IL-17, E = 2.06; IL-1 β , E = 2.08; TNF- α , E = 2.04; RANKL, E = 2.06; all expressed as the fold increase in fluorescence per PCR cycle. The correlation coefficients (R^2) for the PCR dilution series were 0.997 for GAPDH, 0.974 for IL-17, 0.988 for IL-1 β , 0.970 for TNF- α , and 0.990 for RANKL. PCRs for each sample were performed in duplicate. All PCRs were performed in a total volume of 25 μ l with a maximum of 10 ng cDNA per reaction. In addition, we ran nontemplate controls in duplicate for each primer pair of our targeted genes. Relative quantification of the PCR signals was performed by comparing the cycle threshold value (CT), in duplicate, of the gene of interest of each sample with the CT values of the reference gene GAPDH. QPCR analysis for each sample was performed in duplicate.

^{99m}Tc Uptake Measurements

Joint swelling was measured by ^{99m}Tc pertechnetate uptake in the knee joint. This method was shown earlier to correlate well with histological findings. Briefly, mice were sedated with chloralhydrate and subsequently injected intraperitoneally with 20 μ Ci ^{99m}Tc. Thirty minutes thereafter, gamma radiation was assessed by use of a collimated Na-I-scintillation crystal with the knee in a fixed position.

Arthritis was scored as the ratio of the ^{99m}Tc uptake in the right (R) and the left (L) knee joint. R:L ratios >1.1 were taken to indicate swelling of the right knee joint.

Histology

Total knee joints of mice were isolated at day 4 after flare induction. For standard histology, tissue was fixed for 4 days in 4% formaldehyde, decalcified in 5% formic acid, and subsequently dehydrated and embedded in paraffin. Standard frontal sections of 7 μ m were mounted on SuperFrost slides (Menzel-Gläser, Braunschweig, Germany). Hematoxylin and eosin (H&E) staining was performed to study joint inflammation. The severity of inflammation in the knee joints was scored on a scale of 0–3 (0 = no cells, 1 = mild cellularity, 2 = moderate cellularity, and 3 = maximal cellularity). To study proteoglycan (PG) depletion from the cartilage matrix, sections were stained with safranin O followed by counterstaining with fast green. Depletion of PGs was determined using an arbitrary scale of 0 to 3, ranging from normal, fully stained cartilage to destained cartilage, fully depleted of PGs. Bone erosion was graded on a scale of 0 to 5, ranging from no damage to complete loss of the bone structure. Histopathological changes in the knee joint were scored in the patella and femur/tibia regions on five semiserial sections of the joint spaced 70 μ m apart. Scoring was performed in a blindfolded manner by two independent observers.

Cathepsin K Staining

Osteoclast-activity was visualized by immunohistochemistry for cathepsin K. Joint sections (7 μ m) were deparaffinized, rehydrated, and incubated with rabbit-anti-murine cathepsin K (a kind gift of Dr. E. Sakai, Department of Pharmacology, Nagasaki University School of Dentistry, Nagasaki, Japan) or with normal rabbit IgG (X0936; DAKO, Glostrup, Denmark) in phosphate-buffered saline containing 5% milk powder, 3% fetal calf serum, and 2% BSA. Subsequently, the sections were incubated with biotinylated swine-anti-rabbit IgG (E0431; DAKO) followed by labeling with streptavidin-horseradish-peroxidase (P0397; DAKO). Peroxidase was developed with diaminobenzidine as substrate. Sections were counterstained with hematoxylin for 1 minute.

Cytokine Measurements

To determine levels of several cytokines, including IL-1 β , TNF- α , IL-17, and KC in patellae washouts, patellae with surrounding soft tissue consisting of the tendon and synovium were dissected in a standardized manner.¹⁷ Patellae were cultured in RPMI 1640 medium containing 0.1% BSA (200 μ l/patella) for 1 hour at room temperature. Thereafter, supernatant was harvested and centrifuged for 5 minutes at 1000 $\times$ g. Cytokine levels were determined using the Luminex multianalyte technology. The BioPlex system in combination with multiplex cytokine kits (Bio-Rad Laboratories, Hercules, CA) was used. Cyto-

kines were measured in 50 μ l of patellae washout medium. The sensitivity of the multiplex kit was 5, <3, 12.5, and 5 pg/ml, respectively, for IL-1 β , TNF- α , IL-17, and KC.

Statistical Analysis

Differences between experimental groups were tested using the Mann-Whitney *U*-test, unless stated otherwise. *P* values less than 0.05 were considered significant. Results are expressed as the mean $\pm$ SEM.

Results

Pronounced IL-17 Up-Regulation during Antigen-Induced Exacerbation of Experimental Arthritis

Shortly after intraarticular injection of 60 μ g of mBSA in preimmunized mice, a primary chronic joint inflammation develops. During this primary AIA, IL-17 mRNA expression is up-regulated in the synovium at day 1 (Figure 1A) and even more enhanced at days 2 and 7 (Figure 1A). At day 21, IL-17 mRNA expression is low but still present in the synovium of arthritic mice (Figure 1A). Flare-up of arthritis is induced at day 21 by local injection of a small amount of antigen (2 μ g mBSA) into the arthritic knee joint. Interestingly, after antigen rechallenge with only a minor amount of antigen, IL-17 mRNA expression rapidly increases and reaches much higher expression levels than during primary arthritis. A profound increase was already noted after 90 minutes, exceeding the expression levels during primary arthritis, and IL-17 expression peaked after 4 hours with a more than 400-fold increase compared with naive mice (Figure 1A). The expression of IL-17 protein correlated well with mRNA levels, with IL-17 being expressed in low amounts during primary AIA and clearly up-regulated 4 hours after antigen rechallenge (Figure 2B). This rapid and tremendous response during flare reaction suggests involvement of IL-17 produced by activated (memory) T cells located at the site of antigen exposure.

IL-17 Blocking in Exacerbation of Antigen-Induced Arthritis Suppresses Joint Swelling, Inflammation, and Cartilage Depletion

To investigate the importance of IL-17 produced by the synovial infiltrate during the flare-up reaction of arthritis, neutralizing rabbit-anti-mouse-IL-17 antibodies were injected intraperitoneally 2 hours before antigen rechallenge. As a control, normal rabbit antibodies or rabbit-anti-mouse-IL-1 antibodies were given.

Three weeks after induction of primary AIA and just before antigen rechallenge for the induction of a flare reaction, no significant joint swelling was found (99m Tc measurement R/L ratio $\leq$ 1.1; Figure 2A, background). Despite the absence of joint swelling at day 21 of primary

A.

B.

Figure 1. Up-regulation of IL-17 mRNA (A) and protein (B) levels in synovium from arthritic C57Bl/6 mice during AIA. At various time points during primary AIA and flare-up, synovium biopsies were taken for QPCR analysis, and synovial washouts were collected for protein determination. $\Delta\Delta$ CT-value, the mRNA expression corrected for GAPDH and compared with naive mice (day 0). Labels on top of the bars mention the fold mRNA increase compared with naive mice. The QPCR results are the average mRNA expression of two groups, consisting of six pooled synovium biopsies, measured in duplicate. The protein data show the mean with its SEM of three to eight animals per group.

AIA, histological analysis showed some remaining smoldering joint inflammation and cartilage proteoglycan depletion in the background group (Figure 2, B and C). Figure 2D shows representative knee joint sections corresponding to the arbitrary scores for infiltrate and cartilage PG depletion of 0 to 3.

Local injection of 2 μ g mBSA into the hypersensitive joint caused an acute joint swelling at day 1 (Figure 2A). Blocking IL-17 suppressed joint swelling significantly and was even more effective than blocking IL-1 (Figure 2A). Antigen rechallenge caused a marked aggravation of joint inflammation after antigen rechallenge in control-treated mice compared with unchallenged mice (background) (Figure 2B). Neutralizing IL-17 almost completely prevented this exacerbation of synovitis (Figure 2B) and showed comparable effectivity in suppressing joint inflammation as anti-IL-1 treatment. The flare-up reaction also resulted in a marked increase in cartilage PG depletion (Figure 2C). Arthritic mice treated with anti-IL-17 or anti-IL-1 antibodies showed clearly suppressed PG de-

Figure 2. Histopathological effects of anti-IL-17 treatment during flare-up of antigen-induced arthritis. Blocking of IL-17 during flare-up of arthritis results in suppressed joint swelling (**A**), influx of proinflammatory cells (**B**), and cartilage PG depletion (**C**). **D:** Histological sections representative for our arbitrary scoring system for infiltrate and cartilage PG depletion (0–3). Joint swelling was measured by ^{99m}Tc uptake at day 1 of flare-up, and total knee joints were isolated at day 4 for histological analysis. Results are the mean of at least nine mice per group; * $P < 0.05$; ** $P < 0.005$ versus control-treated group, by Mann Whitney *U*-test. Histological sections for infiltrate stained with H&E and for cartilage PG depletion stained with safranin O. P, patella; F, femur; S, synovitis; GP, growth plate; C, cartilage; JS, joint space (Original magnification, $\times 50$ or $\times 100$).

pletion of the cartilage (Figure 2C). These data indicate that blocking IL-17 is effective in preventing synovitis during an arthritic flare reaction and is as potent as blocking IL-1 to prevent cartilage PG depletion.

Neutralizing IL-17 Prevents Bone Erosion and Osteoclast-Like Activity

Histological analysis showed that the increased joint inflammation during flare-up of experimental arthritis is accompanied by marked bone erosions at day 4 (Figure

3A). In addition, immunostaining for cathepsin K, a marker for osteoclast-like activity, showed cathepsin K-positive multinucleate cells at sites of focal bone erosions in knee joints of rechallenged mice (Figure 4A). The degree of bone erosion was significantly suppressed by blocking IL-17 (Figure 3A), and also the osteoclast-like activity was clearly decreased during anti-IL-17 treatment (Figure 4B). Statistical analysis showed a strong correlation between the degree of bone erosions and the severity of joint inflammation during flare-up of AIA (Figure 3B). These findings suggest that neutralizing IL-17 prevents

Figure 3. Effect of anti-IL-17 treatment on bone erosion during flare-up of antigen-induced arthritis. Histological analysis at day 4 of flare-up shows that anti-IL-17 treatment reduces bone erosion in different parts of the murine knee joint (**A**) and that bone erosion is strongly correlated with the degree of joint inflammation (**B**). One group of mice was not flared to obtain background levels of joint pathology before flare-up of arthritis. P, patella; LF, lateral femur; MF, medial femur; Lig, ligament zone; LT, lateral tibia; MT, medial tibia. Results are the mean $\pm$ SEM of at least nine mice per group; * $P < 0.01$; ** $P < 0.005$ versus control-treated group, by Mann Whitney *U*-test. Spearman *R* as calculated to determine the correlation between joint inflammation and bone erosion.

bone erosion by suppressing joint inflammation and osteoclastogenesis at the site of joint inflammation.

Blocking IL-17 Results in Strong Down-Regulation of IL-1 β , TNF- α , and RANKL

To examine the main processes regulated by IL-17 during the flare-up reaction, QPCR analysis on synovial biopsies from control and anti-IL-17-treated mice was performed. Arthritic control mice showed that mRNA expression for proinflammatory cytokines is clearly up-regulated at day 1 of flare-up (Table 1): IL-1 β expression was increased more than 20-fold (4.4 PCR cycles), TNF- α circa fivefold (2.3 cycles), and IL-17 almost 40-fold (5.2 cycles). Antigen rechallenge also resulted in up-regulation of the osteoclast-differentiation factor RANKL for more than fivefold (2.4 PCR cycles) (Table 1). Anti-IL-17 treatment during flare-up almost completely prevented the up-regulation of IL-1 mRNA (Table 1), and cytokine analysis in synovial wash-outs of arthritic mice confirmed that IL-1 levels were significantly reduced by both anti-IL-17 and anti-IL-1 treatment (Figure 5A).

Interestingly, whereas IL-1 blocking hardly affected TNF expression, blocking IL-17 also reduced TNF- α mRNA and (although not significantly) TNF- α protein

Figure 4. Anti-IL-17 treatment during flare-up of experimental arthritis reduces cathepsin K staining as a marker for osteoclast-like activity. Arthritic mice were treated with control rabbit antibodies (**A**) or anti-IL-17 antibodies (**B**), and total knee joints were isolated at day 4 for histological analysis. F, femur; C, cartilage; S, synovium. Original magnification, $\times 100$ and $\times 200$. Pictures are representative of immunostainings on several joint sections of both groups.

level (Table 1, Figure 5B), suggesting that TNF- α acts downstream of IL-17 during arthritic flares. Arthritic mice treated with anti-IL-17 antibodies also showed a trend for suppressed KC expression (Figure 5C), the murine equivalent of interleukin-8, and an important chemotactic factor for neutrophil recruitment. These data suggest that in addition to the proinflammatory capacity of IL-17 itself, this T-cell cytokine is an important upstream mediator driving the expression of TNF- α , IL-1 β , RANKL, and KCs during reactivation of joint inflammation.

Discussion

In the present study, we clearly demonstrated the critical role of IL-17 in driving joint inflammation and

Table 1. QPCR Results of Cytokine mRNA Levels in Murine Synovium at Day 1 of Flare of AIA with or without Anti-IL-17/IL-1 Treatment

	$\Delta\Delta\text{CT}$ (fold increase)			
	IL-1 β	TNF α	IL-17	RANKL
Control	4.4 (21)	2.3 (4.9)	5.2 (37)	2.4 (5.3)
Anti-IL-17	0.7 (1.6)	1.0 (2.0)	0 (0)	-1.2 (-2.3)
Anti-IL-1	3.6 (12)	2.8 (7.0)	3.4 (11)	0.8 (1.7)

C57Bl/6 mice were immunized and boosted with mBSA/CFA, and arthritis was induced by injecting 60 μg of mBSA. At week 3 of arthritis, 2 μg of mBSA was injected into the arthritic joint to induce a flare-up. Two hours before the induction of flare-up, arthritic mice received anti-IL-17 antibodies, anti-IL-1 antibodies, or control rabbit antibodies. One group was not injected with antigen to collect background levels of mRNA expression before flare-up of arthritis. At day 1 of flare-up, synovium biopsies were taken for quantitative PCR analysis. mRNA levels were determined for various proinflammatory cytokines. Expression levels were corrected for GAPDH and are shown in this table as $\Delta\Delta\text{CT}$ value, which is the mRNA expression level (in PCR cycles) compared with the control-treated group. The QPCR results are the average mRNA expression of two groups, consisting of six pooled synovium biopsies, measured in duplicate.

cartilage depletion during flare-up of experimental arthritis. In addition, neutralizing IL-17 during antigen rechallenge prevents bone erosion and results in suppressed synovial expression of proinflammatory cytokines and chemokines.

The proinflammatory IL-17, mainly produced by activated (memory) T cells, is a likely contributor to the pathogenesis of rheumatoid arthritis. IL-17 is found in the synovium and synovial fluid of RA patients,²²⁻²⁴ and its proinflammatory and catabolic properties have been studied extensively both *in vitro* and in experimental arthritis. IL-17 stimulates the release of IL-1 and TNF by human macrophages²⁵ and enhances the production of

IL-6 and IL-8 by synovial fibroblasts.²⁶ Both *in vitro* and *in vivo* experiments have shown cartilage breakdown by IL-17,²⁷ effects that become more pronounced in combination with IL-1 or TNF.²⁸ Blocking of endogenous IL-17 in the autoimmune CIA model showed that IL-17 plays a role in early stages of arthritis, but also later during disease progression.^{17,18} Furthermore, the spontaneous development of arthritis normally found in mice lacking IL-1Ra did not occur after crossing IL-17-deficient mice with IL-1Ra-deficient mice.¹⁹ Our recent study in mice deficient for IL-17R strongly suggested IL-17/IL-17R signaling to be a pivotal mechanism in turning a nondestructive macrophage-driven joint inflammation into a chronic T-cell-mediated destructive synovitis (unpublished data). These and other studies on IL-17 confirm the important role of this T-cell cytokine in onset and progression of arthritis. The novel findings in the present study show a critical role for IL-17 in the flare-up reaction of experimental arthritis, driving joint inflammation and bone erosion by regulating cytokine and chemokine expression.

In the model of AIA, flare-up can be induced in the arthritic knee joint by the local injection of a small amount of antigen (mBSA). This reactivation of a smoldering arthritis results in joint swelling, inflammation, and joint destruction. Previous studies in AIA-flare showed that T cells and the activation of T cells by antigen-presenting cells are required for flare-up of experimental arthritis.^{9,29} In our previous study with anti-IL-1 treatment in AIA-flare, IL-1 was found to be mainly involved in cartilage destruction. Joint swelling and early cell influx were for the greater part not IL-1-mediated,¹² suggesting that other mediators are involved in these processes. In the present study, we selectively blocked the T-cell cytokine IL-17 with antibodies, and this markedly reduced joint swelling and inflammation during the antigen-induced flare-up. Furthermore, cartilage proteoglycan depletion was suppressed in the anti-IL-17-treated mice compared with the control group. Because IL-17 is highly up-regulated early after flare-up induction, neutralizing IL-17 protein prevents influx of inflammatory cells. This includes IL-17-producing activated T cells, leading to indirect down-regulation of mRNA expression of IL-17 in the synovium further on during the flare-up as was measured at day 1.

Besides the proinflammatory capacity of IL-17 itself, this T-cell cytokine plays an important role in driving synovial expression of other proinflammatory cytokines and chemokines. QPCR and cytokine analysis showed suppressed expression of IL-1, TNF- α , and KCs in mice treated with anti-IL-17 antibodies, suggesting that IL-17 acts as an upstream mediator driving joint inflammation. Anti-IL-17 treatment clearly reduced synovial TNF- α expression and completely prevented joint swelling, underlining the involvement of TNF- α in this process.³⁰ In contrast, blocking IL-1 also resulted in reduced influx of inflammatory cells but failed to suppress joint swelling, possibly by lacking a reduction in TNF- α expression. Interestingly, our results also indicate that TNF- α acts downstream of IL-17 in the arthritis flare-up process and is not regulated by IL-1.

Down-regulation of IL-17 and its downstream mediators by anti-IL-17 treatment is probably not the only pro-

Figure 5. Neutralizing IL-17 or IL-1 during flare-up of arthritis reduces the levels of cytokines and chemokines in synovial washouts of arthritic mice. At various times after flare-induction, synovial washouts were collected and subsequently analyzed for cytokine and chemokine levels; IL-1 β at 90 minutes and day 1 of flare-up (A); TNF- α at 90 minutes (B), and KCs at day 1 (C). Arthritic mice were treated with anti-IL-17, anti-IL-1, or control antibodies. One group of mice was not treated to obtain background expression levels before flare-up of arthritis. Results are the mean of five samples per group ($n = 3$ for background); * $P < 0.05$; ** $P < 0.01$ versus control-treated group, by Mann Whitney *U*-test.

cess by which joint inflammation is suppressed. Previous *in vitro* and *ex vivo* studies have shown the great capacity of IL-17 to synergize with TNF- α and IL-1 in the induction of other cytokines and chemokines^{31,32} and the degradation of cartilage^{33,34} and bone.³⁵ The interaction of IL-17 with other cytokines during reactivation of experimental arthritis, in particular the additive and synergistic effects with TNF- α and IL-1, might play an important role in preventing joint pathology.

Synovial fluids from RA patients often contain clearly elevated levels of IL-17.^{23,24} This study demonstrated that antigen rechallenge in the arthritic joint induced marked bone erosion, and blocking of IL-17 was found to be highly effective in preventing this form of joint destruction. Previous anti-IL-17 treatment during rat adjuvant arthritis has also shown significant suppression of radiological scores.³⁶ These findings suggest a possible beneficial effect of anti-IL-17 treatment in RA patients in the prevention of further bone damage during arthritic flares. The protective effect of neutralizing endogenous IL-17 reported here was supported by reduced synovial RANKL and cathepsin K expression. In this study, we found a strong correlation between the degree of bone erosion and the severity of joint inflammation. In the anti-IL-17-treated mice, the correlation between inflammation and bone erosion remained. Our findings suggest that the prevention of bone erosion by anti-IL-17 treatment can for a greater part be attributed to effective suppression of joint inflammation.

Data from the present study demonstrate the critical role of IL-17 in driving joint inflammation and cartilage depletion during flare-up of experimental arthritis. In addition, neutralizing IL-17 during antigen rechallenge prevents bone erosion and results in suppressed synovial expression of proinflammatory cytokines and chemokines. Because neutralizing IL-17 has been shown to be remarkably effective in this experimental model of arthritis exacerbation, blocking of IL-17 might be an interesting therapeutic target in the treatment of antigen-specific relapses during RA.

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