ARTHRITIS & RHEUMATISM
Vol. 58, No. 11, November 2008, pp 3461–3470
DOI 10.1002/art.23957

Interleukin-1 Drives Pathogenic Th17 Cells During Spontaneous Arthritis in Interleukin-1 Receptor Antagonist–Deficient Mice

Marije I. Koenders,[1] Isabel Devesa,[1] Renoud J. Marijnissen,[1] Shahla Abdollahi-Roodsaz,[1] Annemieke M. H. Boots,[2] Birgitte Walgreen,[1] Franco E. di Padova,[3] Martin J. H. Nicklin,[4] Leo A. B. Joosten,[1] and Wim B. van den Berg[1]

Objective. **Interleukin-1 receptor antagonist–deficient (IL-1Ra$^{-/-}$) mice spontaneously develop an inflammatory and destructive arthritis due to unopposed excess IL-1 signaling. In this study, the role of Th17 cells and the effect of neutralization of IL-17, IL-1, and tumor necrosis factor α (TNFα) were investigated in this IL-1–driven murine arthritis model.**

Methods. **T cells isolated from IL-1Ra$^{-/-}$ and wild-type (WT) mice were stained for IL-17 and interferon-γ, with results assessed by fluorescence-activated cell sorting analysis. To investigate the contribution of IL-1 and IL-17 in further progression of arthritis in this model, mice were treated with neutralizing antibodies after the onset of arthritis.**

Results. **Compared with WT mice, IL-1Ra$^{-/-}$ mice had similar levels of Th1 cells but clearly enhanced levels of Th17 cells; this increase in the number of Th17 cells was evident even before the onset of arthritis, in young, nonarthritic IL-1Ra$^{-/-}$ mice. The percentage of Th17 cells increased even more after the onset of arthritis and, similar to the serum levels and local messenger RNA levels of IL-17, the percentage of IL-17+ Th17 cells clearly correlated with the severity of arthritis. Anti–IL-17 treatment prevented any further increase in inflammation and bone erosion, whereas blocking of TNFα after the onset of arthritis had no effect. In contrast, neutralization of IL-1 resulted in a complete suppression of arthritis. Interestingly, this anti–IL-1 treatment also significantly reduced the percentage of IL-17+ Th17 cells in the draining lymph nodes of these arthritic mice.**

Conclusion. **Increased levels of Th17 cells can be detected in IL-1Ra$^{-/-}$ mice even preceding the onset of arthritis. In addition, the results of cytokine-blocking studies demonstrated that IL-17 contributes to the inflammation and bone erosion in this model, which suggests that IL-1 is the driving force behind the IL-17–producing Th17 cells.**

Rheumatoid arthritis (RA) is an autoimmune disease with unknown etiology, characterized by progressive inflammation and destruction of multiple joints. Cytokines are important mediators in the arthritic process, driving the synovial inflammation and contributing, directly or indirectly, to degradation of cartilage and bone. Tumor necrosis factor α (TNFα) and interleukin-1 (IL-1) are considered to be key cytokines in the RA process (1,2), and their actions can be effectively blocked with biologic agents such as etanercept (Enbrel), infliximab (Remicade), adalimumab (Humira), and anakinra (Kineret) (2). Despite the very good results obtained with these biologic agents in the treatment of RA, not all RA patients respond well to these therapies, although the reason for this nonresponsiveness is unclear. Immune reactions against the biologic

Dr. Koenders' work was supported by a grant from Novartis Pharma AG (Basel, Switzerland). Dr. Devesa's work was supported by a grant from the Spanish Ministry of Education and Science.

[1]Marije I. Koenders, PhD, Isabel Devesa, PhD, Renoud J. Marijnissen, MSc, Shahla Abdollahi-Roodsaz, MSc, Birgitte Walgreen, BSc, Leo A. B. Joosten, PhD, Wim B. van den Berg, PhD: Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands; [2]Annemieke M. H. Boots, PhD: Organon NV, Oss, The Netherlands; [3]Franco E. di Padova, MD, PhD: Novartis Institutes for Biomedical Research, Basel, Switzerland; [4]Martin J. H. Nicklin, PhD: University of Sheffield, Sheffield, UK.

Drs. Koenders and Devesa contributed equally to this work.

Address correspondence and reprint requests to Marije I. Koenders, PhD, Radboud University Nijmegen Medical Center, Department of Rheumatology, Rheumatology Research and Advanced Therapeutics, 272, Geert Grooteplein 28, PO Box 9101, 6500 HB Nijmegen, The Netherlands. E-mail: m.koenders@reuma.umcn.nl.

Submitted for publication January 29, 2008; accepted in revised form July 7, 2008.

agent or a limited half-life of the compound can result in suboptimal blocking of the targeted cytokine (3,4). In addition, RA is a widely heterogeneous disease, indicating that the relative role of a specific cytokine can have great individual variation.

Animal models of arthritis offer the possibility of studying the relative role of specific cytokines in a simplified setup. IL-1 receptor antagonist–deficient (IL-1Ra$^{-/-}$) mice form an elegant model of an IL-1–driven experimental arthritis in which the role of a variety of cytokines in the arthritic process can be studied. In IL-1Ra$^{-/-}$ mice, the absence of IL-1Ra results in spontaneous arthritis due to excess IL-1 signaling (5). Crossing of these mice with TNF$\alpha^{-/-}$ mice results in a crossbred strain that exhibits reduced incidence and severity of spontaneous arthritis (6), indicating that TNFα is important in the development of arthritis in this model.

In a study using 2 different approaches (6), it was shown that T cells are also very important in this arthritis model, since spontaneous arthritis did not develop when the mice had no functional T (and B) cells, and arthritis could be transferred by T cells to naive nude mice (6). In another study, IL-1Ra$^{-/-}$ mice showed increased levels of IL-17 after stimulation of splenic T cells (7), and thus the role of IL-17 in this model was explored by crossing the IL-1Ra$^{-/-}$ mice with IL-17$^{-/-}$ mice; this additional IL-17 deficiency completely blocked the onset of the disease (7), indicating a crucial role for IL-17 in the development of arthritis in this murine model.

IL-17 is a proinflammatory cytokine that is present in the synovial tissue and synovial fluid of RA patients (8–12). IL-17 has always been regarded as a cytokine that is mainly produced by activated T cells, but it was only recently assigned its own unique T cell subset, the Th17 cells (13,14). Th17 cells originate from naive precursor T helper cells and are driven by various cytokines, such as IL-6 and transforming growth factor β, IL-23, and IL-1 (15–17). Th17 cells express the specific transcription factor retinoic acid–related orphan receptor γT (18) and are characterized by the selective production of IL-17 (19). IL-17 can induce expression of IL-1 and TNFα (19), but can also act on inflammation and destruction independent of these cytokines (20,21). IL-17 has been shown to contribute to inflammation and destruction in vitro (22–24) and in mouse models (25–28), and promising effects of IL-17 blockade in animal models of arthritis have resulted in the first clinical trials with neutralizing anti–IL-17 antibodies.

Blocking of IL-17 in mice with collagen-induced arthritis (CIA) reduced the inflammation and destruction of the joints, even in the late stages after onset of the disease (29), and effects were even more pronounced when IL-17 was neutralized in the T cell–driven flare reaction of antigen-induced arthritis (30). In this study, we investigated the role of Th17 cells and the effect of neutralization of IL-17, relative to that of IL-1 or TNFα, in the progression of a strongly IL-1–driven experimental arthritis, by blocking these cytokines after the onset of arthritis in IL-1Ra$^{-/-}$ mice.

MATERIALS AND METHODS

Animals. IL-1Ra$^{-/-}$ mice on the BALB/c background were kindly supplied by Dr. M. Nicklin (Sheffield, UK), and were generated as described previously (31). The IL-1Ra$^{-/-}$ mice were backcrossed onto the arthritis-susceptible strain BALB/c for at least 8 generations; BALB/c mice from Charles River (Wilmington, MA) were used as wild-type (WT) controls. All mice were housed in filter-top cages under specific pathogen–free conditions, and a standard diet and water were provided ad libitum. The mice used for antibody treatment were between 10 weeks and 14 weeks of age. All animal procedures were approved by the institutional ethics committee.

Arthritis score. The clinical severity of arthritis (arthritis score) was macroscopically graded on a scale of 0–2 for each paw (nonarthritic = macroscopic score 0, mild arthritis = macroscopic score 0.25–1.25, severe arthritis = macroscopic score 1.5–2.0). This macroscopic grading system assessed the extent of changes in redness and swelling of the paws.

Cytokine measurements. To determine levels of the cytokines IL-1β, TNFα, and IL-17 in serum samples, Luminex multianalyte technology was used in combination with multiplex cytokine kits (Bio-Rad, Hercules, CA). Cytokines were measured in 25 μl of serum, diluted 1:3 in serum diluent (Bio-Rad). The sensitivity of the multiplex kit was $<$3 pg/ml.

Fluorescence-activated cell sorter (FACS) staining. CD3+ T cells from the spleens of mice were isolated using negative selection with a magnetic sorter and microbeads from the Pan T Cell isolation kit (Miltenyi Biotec, Sunnyvale, CA). The purity of the CD3+ T cells was $>$95%. These isolated T cells from the spleen and lymph nodes were stimulated with 50 ng/ml phorbol myristate acetate and 1 μg/ml ionomycin in the presence of GolgiPlug (catalog no. BD555029) for 5 hours, and then stained with anti-CD4 and fixed and permeabilized using BD Cytofix/Cytoperm (BD Biosciences, San Jose, CA), followed by intracellular staining with anti–IL-17 and anti–interferon-γ (anti-IFNγ). The antibodies used were allophycocyanin-labeled rat anti-CD4 IgG2a (catalog no. BD553051), phycoerythrin-labeled rat anti–IL-17 IgG1 (catalog no. BD559502), fluorescein isothiocyanate–labeled rat anti-IFNγ IgG1 (catalog no. BD554411), and corresponding isotype controls (all from BD Biosciences).

Study protocol. IL-1Ra$^{-/-}$ mice with early (established) arthritis (defined as having an arthritis score of 0.75–1.0) were treated with different cytokine blockers. Mice received anticytokine treatment intraperitoneally on day 0 and day 4 of the study, and the joints were scored macroscopically

for arthritis on days 2, 4, and 7. On day 7, serum samples were obtained and ankle joints were isolated for histologic analysis. In addition, the spleen and lymph nodes were isolated for FACS analysis.

Anticytokine treatments. IL-17 was neutralized with a rat anti-mouse IL-17 monoclonal antibody (catalog no. MAB421; R&D Systems, Minneapolis, MN). IL-1β was neutralized with a rat anti-mouse IL-1β monoclonal antibody (1400.24.17, MM425; Perbio Science, Bonn, Germany). Injections were given intraperitoneally. As a control, the same amount of rat isotype control antibodies was injected. To neutralize TNFα, mice were treated with dimerically linked PEGylated soluble p55 TNF receptor I (TNFRI; Amgen, Boulder, CO) at a dose of 3 mg/kg or 10 mg/kg. Efficacy of this TNFRI, referred to as TNFbp, has previously been observed in murine streptococcal cell wall–induced arthritis (32) and CIA (33).

Histology. For standard histologic assessment, isolated joints were fixed for 4 days in 10% formalin, decalcified in 5% formic acid, and subsequently dehydrated and embedded in paraffin. Standard frontal sections (7 μm) of the joint tissue were mounted on SuperFrost slides (Menzel-Gläser, Braunschweig, Germany). Hematoxylin and eosin staining was performed to study joint inflammation. The severity of inflammation in the 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 PG was determined using an arbitrary scale of 0–3, ranging from normal, fully stained cartilage to destained cartilage that was fully depleted of PG. Bone destruction was graded on a scale of 0–3, ranging from no damage to the complete loss of bone structure. Histopathologic changes were scored on 5 semiserial sections of the joint, with sections spaced 70 μm apart. Scoring was performed in a blinded manner by 2 independent observers.

IL-1β immunostaining. Tissue sections (7 μm) were deparaffinized, rehydrated, and treated with 3% H_2O_2 for 10 minutes at room temperature. Sections were incubated for 12 minutes in 10 m*M* of warm citrate buffer (pH 6.0), and thereafter incubated for 1 hour with rabbit anti-mouse IL-1β antibodies (H-135) (catalog no. sc-7884; Santa Cruz Biotechnology, Santa Cruz, CA) or irrelevant primary isotype-specific IgG antibodies. After rinsing, sections were incubated for 30 minutes with biotinylated swine anti-rabbit antibodies (E0431; Dako, Carpinteria, CA), followed by labeling with streptavidin–horseradish peroxidase (HRP) (P0397; Dako). Peroxidase was developed with diaminobenzidine (DAB) as substrate. Sections were counterstained with hematoxylin for 1 minute.

Cathepsin K immunostaining. Osteoclast activity was visualized by immunohistochemical analysis for cathepsin K. Joint sections (7 μm) were deparaffinized, rehydrated, and incubated with rabbit anti-mouse cathepsin K (a kind gift from Dr. E. Sakai, Department of Pharmacology, Nagasaki University School of Dentistry, Nagasaki, Japan) or with normal rabbit IgG (X0936; Dako) in phosphate buffered saline containing 5% milk powder, 3% fetal calf serum, and 2% bovine serum albumin. Subsequently, the sections were incubated with biotinylated swine anti-rabbit IgG (E0431; Dako), followed by labeling with streptavidin–HRP (P0397; Dako). Peroxidase was developed with DAB as substrate. Sections were counterstained with hematoxylin for 1 minute.

Statistical analysis. Results are expressed as the mean ± SEM. Differences between experimental groups were tested using the Mann-Whitney U test or one-way analysis of variance with Dunnett's multiple comparison test, as appropriate. Correlations were determined using Spearman's correlation coefficients. P values less than 0.05 were considered significant.

RESULTS

Development of arthritis in IL-1Ra–deficient mice. IL-1Ra$^{-/-}$ mice on the BALB/c background developed spontaneous arthritis, especially in the ankle joints of the hind paws (Figure 1). In our colony of IL-1Ra$^{-/-}$ mice (n = 95), arthritis started to develop at the age of 3 weeks. At 16 weeks of age, >70% of the animals had at least 1 arthritic joint. The incidence of arthritis in the front paws was very low, with only 4% of the mice having 1 or both front paws affected. At 16 weeks of age, 45% of the mice had developed symmetric arthritis, with both hind paws affected (Figure 1A). The mean period between arthritis onset in 1 hind paw and the development of symmetric arthritis in both hind paws was 1.5 weeks (range 0–8 weeks).

Increase in serum and synovial cytokine levels in arthritic IL-1Ra–deficient mice. Serum and synovial biopsy samples from arthritic mice and WT BALB/c mice were collected to determine the systemic and local expression levels of IL-1, TNFα, and IL-17. First, the serum levels of these cytokines were analyzed using Luminex bead array, and subsequently these expression levels were assessed for correlations with age.

As shown in Figure 1C, the serum levels of IL-1, IL-17, and TNFα clearly increased during the development of spontaneous arthritis in IL-1Ra$^{-/-}$ mice. In contrast, serum levels in WT BALB/c mice did not increase or hardly increased over time (Figure 1B). The results of Spearman's correlation analyses showed that the serum levels of all 3 cytokines significantly correlated with the age of the IL-1Ra$^{-/-}$ mice (for IL-1, r = 0.810, $P < 0.00001$; for IL-17, r = 0.521, $P = 0.0032$; for TNFα, r = 0.479, $P = 0.0074$).

In addition to determining the systemic levels of these cytokines, synovial biopsy specimens were collected from the knee and ankle joints of IL-1Ra$^{-/-}$ mice with various degrees of arthritis to assess the expression of all 3 cytokines. The local messenger RNA (mRNA)

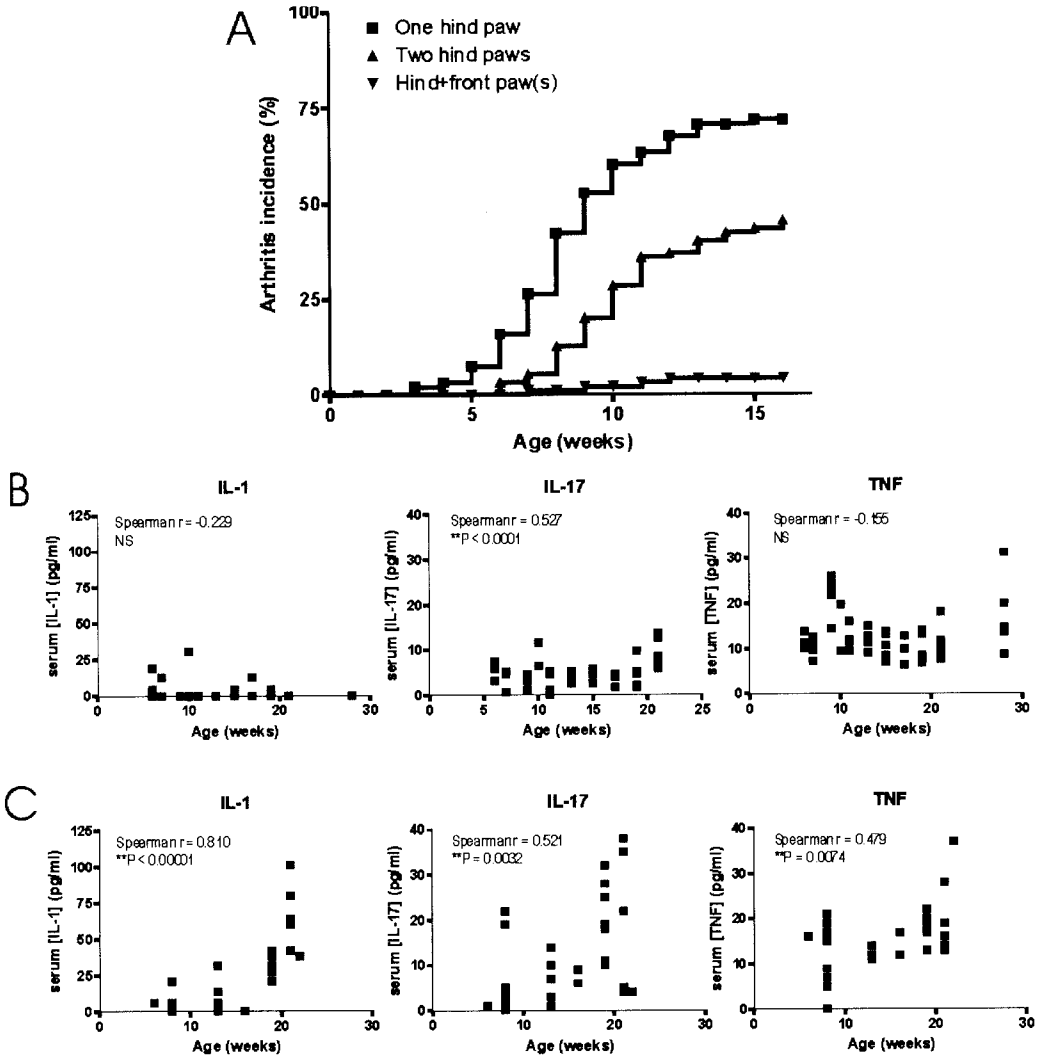

Figure 1. Incidence of arthritis **(A)** and correlations of serum cytokine levels with age **(B** and **C)** in interleukin-1 receptor antagonist–deficient (IL-1Ra$^{-/-}$) mice. Development of arthritis was inspected weekly in different joints of IL-1Ra$^{-/-}$ mice (n = 95), and the percentage of arthritic mice was determined **(A)**. Arthritis was more common in the ankle (hind paw) joints than in the front paws, and was expressed symmetrically in the hind paws in almost two-thirds of the arthritic mice. Serum levels of IL-1β, IL-17, and tumor necrosis factor α (TNFα) were determined in wild-type BALB/c mice **(B)** and in arthritic IL-1Ra$^{-/-}$ mice **(C)**, with correlations between serum cytokine levels and age expressed as Spearman's correlation coefficients. Squares in **B** and **C** represent individual serum samples. ** = significant *P* value. NS = not significant.

expression of IL-1, IL-17, and TNFα was determined using real-time quantitative polymerase chain reaction (PCR); the results, as shown in Figure 2, are the relative mRNA expression, determined in relation to the levels of GAPDH.

In comparison with the IL-1 mRNA levels in the synovium of noninflamed knee or ankle joints, IL-1 mRNA levels in arthritic synovium were tremendously up-regulated (Figure 2A), with an increase of 9–13 PCR cycles. A marked up-regulation was also found for IL-17 mRNA expression in arthritic synovium, which showed an increase of 8–13 PCR cycles as compared with that in nonarthritic synovium (Figure 2B). Although not as impressive as the findings for IL-1 or IL-17, an up-regulation of 5–7 PCR cycles was found for TNFα mRNA expression (Figure 2C). In

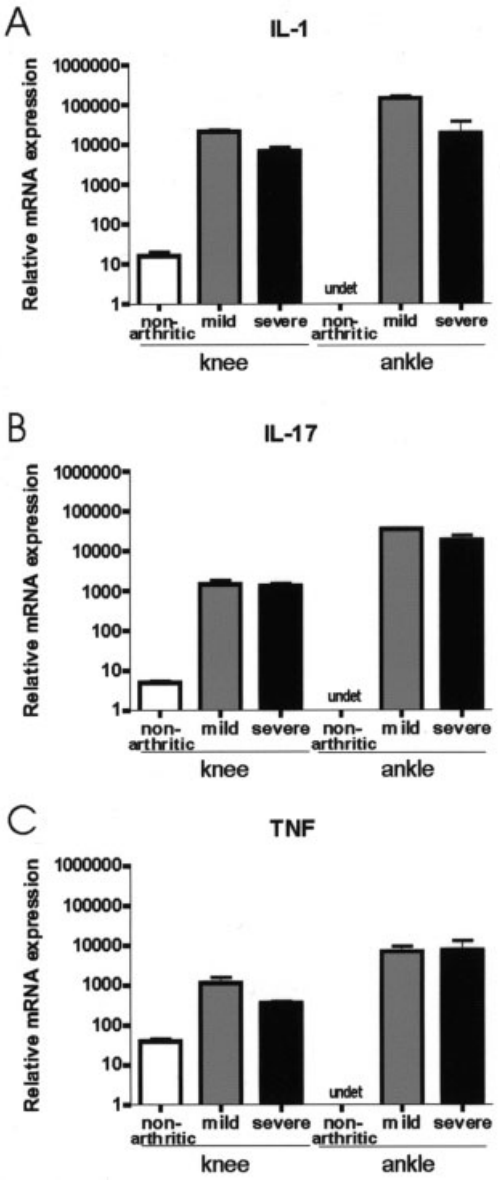

Figure 2. Messenger RNA expression of IL-1 (**A**), IL-17 (**B**), and TNFα (**C**) in synovial biopsy samples from nonarthritic and arthritic knee and ankle joints of IL-1Ra$^{-/-}$ mice. Joints were categorized as nonarthritic (macroscopic score 0) or arthritic (mild = macroscopic score 0.25–1.25, severe = macroscopic score 1.5–2.0). Results are the relative mRNA expression, determined in relation to GAPDH levels and calculated as $2^{-\Delta\Delta C_t} \times 10^6$. Bars show the mean and SEM results of 2 experiments with pooled biopsy samples from at least 3 mice per group. Undet = undetectable expression after 40 polymerase chain reaction cycles (see Figure 1 for other definitions).

WT BALB/c synovium, mRNA levels for these 3 cytokines were below the limit of detection. These expression data show that increasing amounts of IL-1, IL-17, and TNFα can be found both systemically and locally during the development of arthritis in IL-1Ra$^{-/-}$ mice, suggesting a role for these cytokines in the inflammatory and destructive processes in this arthritis model.

Increased numbers of Th17 cells during arthritis development in IL-1Ra$^{-/-}$ mice. Since high levels of IL-17 were found both systemically and locally in the arthritic IL-1Ra$^{-/-}$ mice, we next investigated the most likely source of this cytokine: the IL-17–producing Th17 cell. T cells were isolated from draining lymph nodes and spleens to study the Th17 (and Th1) cells during the progression of arthritis. T cells were isolated from WT BALB/c mice and from IL-1Ra$^{-/-}$ mice with various degrees of clinical severity of arthritis (nonarthritic, mild arthritis, or severe arthritis). The cells were stained for expression of IL-17 and IFNγ, with results assessed by FACS analysis.

The percentage of IFNγ+ Th1 cells in the lymph nodes and spleens was comparable between the IL-1Ra$^{-/-}$ and WT mice, and these levels did not increase

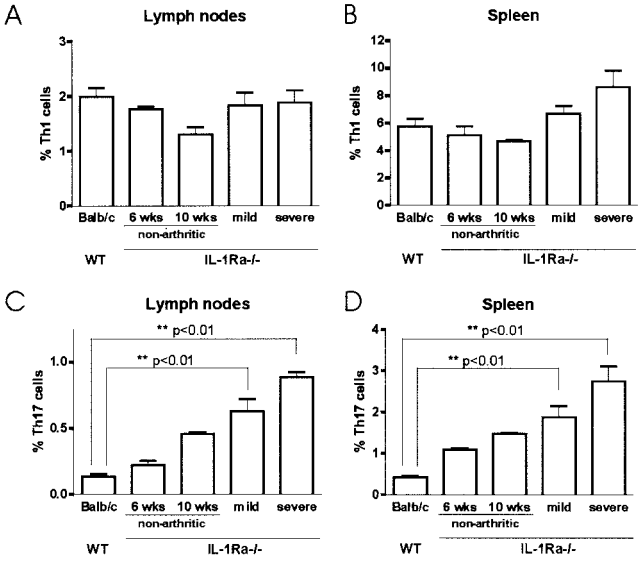

Figure 3. Fluorescence-activated cell sorter analysis of Th1 (**A** and **B**) and Th17 (**C** and **D**) cell subsets in isolated lymphocytes from the lymph nodes (**A** and **C**) and spleens (**B** and **D**) of nonarthritic and arthritic interleukin-1 receptor antagonist–deficient (IL-1Ra$^{-/-}$) mice and wild-type (WT) BALB/c mice. The percentage of Th1 cells remained stable during the development of arthritis in IL-1Ra$^{-/-}$ mice, whereas that of Th17 cells increased with time and severity of arthritis. Joints of IL-1Ra$^{-/-}$ mice were categorized as nonarthritic (macroscopic score 0) or arthritic (mild = macroscopic score 0.25–1.25, severe = macroscopic score 1.5–2.0). Groups were compared by one-way analysis of variance with Dunnett's multiple comparison test. Bars show the mean and SEM percentage of Th1 or Th17 cells.

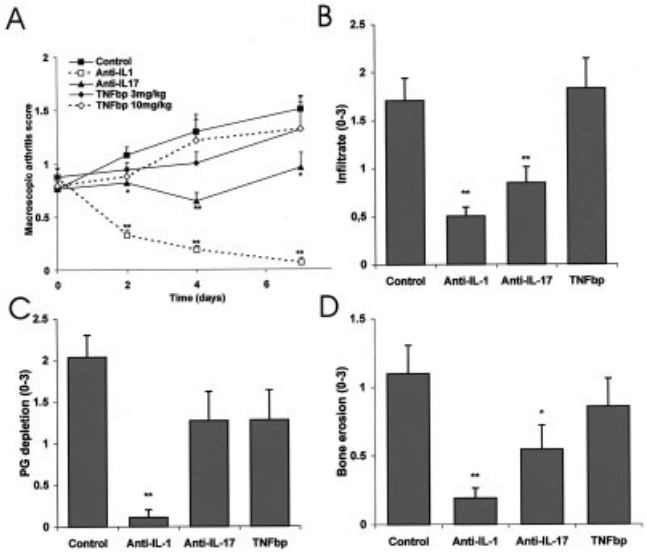

Figure 4. Macroscopic arthritis scores (scale 0–2) **(A)** and histologic scores for inflammation **(B)**, cartilage proteoglycan (PG) depletion **(C)**, and bone erosion **(D)** (scale 0–3 for all) in the arthritic ankle joints of interleukin-1 receptor antagonist–deficient (IL-1Ra$^{-/-}$) mice treated with anti–IL-1, anti–IL-17, 2 different doses (3 mg/kg and 10 mg/kg) of PEGylated soluble p55 tumor necrosis factor receptor I (TNFbp), or control antibodies. Scores for inflammatory cell infiltration **(B)**, cartilage PG depletion **(C)**, and bone erosion **(D)** were assessed 7 days after the start of treatment. Results are the mean and SEM of at least 8 mice per group. $* = P < 0.05$; $** = P < 0.01$, versus control antibody–treated group, by Mann-Whitney U test.

significantly during arthritis progression (Figures 3A and B). In contrast, IL-1Ra$^{-/-}$ mice showed increasing percentages of IL-17+ Th17 cells with further progression of arthritis, in both the lymph nodes and the spleens, and even nonarthritic IL-1Ra$^{-/-}$ mice had significantly higher IL-17+ Th17 levels than did WT BALB/c mice (combined data from 6- and 10-week-old nonarthritic mice versus WT mice, $P = 0.0144$ in the lymph nodes, $P < 0.0001$ in the spleens) (Figures 3C and D).

Suppression of disease severity by blocking of endogenous IL-17 in arthritic IL-1Ra–deficient mice. To study the role of IL-17 in comparison with that of IL-1 and TNFα in the progression of spontaneous arthritis in this murine model, IL-1Ra$^{-/-}$ mice with early (established) arthritis (arthritis score of 0.75–1.0) were treated with blockers for IL-1, IL-17, or TNFα. Mice received anticytokine treatment intraperitoneally on day 0 and day 4 of the study, and joints were scored macroscopically for arthritis on days 2, 4, and 7. On day 7, the ankle joints were isolated for histologic analysis. As shown in Figure 4A, neutralization of TNFα after the first clinical signs of arthritis had no effect on arthritis severity, even after increasing the dose of TNFbp from 3 mg/kg to 10 mg/kg. This indicates that, despite the previously demonstrated role of TNFα in the development of IL-1Ra$^{-/-}$ arthritis, TNFα is not important during the progression of this arthritis in mice.

In contrast, blocking of IL-17 clearly halted further progression of this T cell–driven arthritis, although neutralization of IL-17 did not result in a decrease in the macroscopic arthritis score (Figure 4A). This finding is in contrast to that obtained with anti–IL-1 treatment in arthritic IL-1Ra$^{-/-}$ mice, in which an impressive disappearance of the macroscopic signs of arthritis was observed after blocking of IL-1 (Figure 4A). These results suggest that IL-17 is crucial not only at the onset of disease, as was demonstrated by crossing IL-1Ra$^{-/-}$ with IL-17$^{-/-}$ mice (7), but also in the progression of this T cell–driven arthritis in mice. However, despite the role of T cells and IL-17, IL-1 remains the driving factor in this arthritis model.

Reduction of joint inflammation and bone erosion by neutralization of IL-17. Ankle joints of IL-Ra$^{-/-}$ mice isolated on day 7 of the study were processed for histologic analysis and assessed for inflammation and destruction. Consistent with the lack of change in macroscopic arthritis scores, anti-TNFα treatment after the onset of arthritis also did not result in a suppression of inflammation, a decrease in cartilage PG depletion, or a reduction in bone erosion (Figures 4B–D). In contrast, neutralization of IL-1 after the onset of arthritis was very effective in suppressing joint inflammation, and also markedly reduced cartilage PG depletion and bone erosion (Figures 4B–D).

Blocking of IL-17 not only halted macroscopic inflammation (Figure 4A), but also significantly suppressed the influx of proinflammatory cells detected on histologic analysis (Figure 4B). Cartilage PG depletion was not significantly suppressed by blocking of IL-17 (Figure 4C), but anti–IL-17 treatment did clearly reduce bone erosions (Figure 4D). This effect of blocking of IL-17 on inflammation and bone erosion was accompanied by significantly reduced expression of IL-1 and cathepsin K, a marker of osteoclast activity, in the arthritic joints (Figures 5A–F). This suggests that IL-17 partially contributes to inflammation and destruction of the joints in arthritic IL-1Ra$^{-/-}$ mice, by acting upstream of local IL-1 and enhancing synovial expression of IL-1 and osteoclast differentiation.

Reduction of IL-17+ Th17 cells by anti–IL-1 treatment. Since anti–IL-1 treatment is very powerful in this IL-1–driven model, and the levels of Th17 cells clearly correlated with arthritis severity, we next inves-

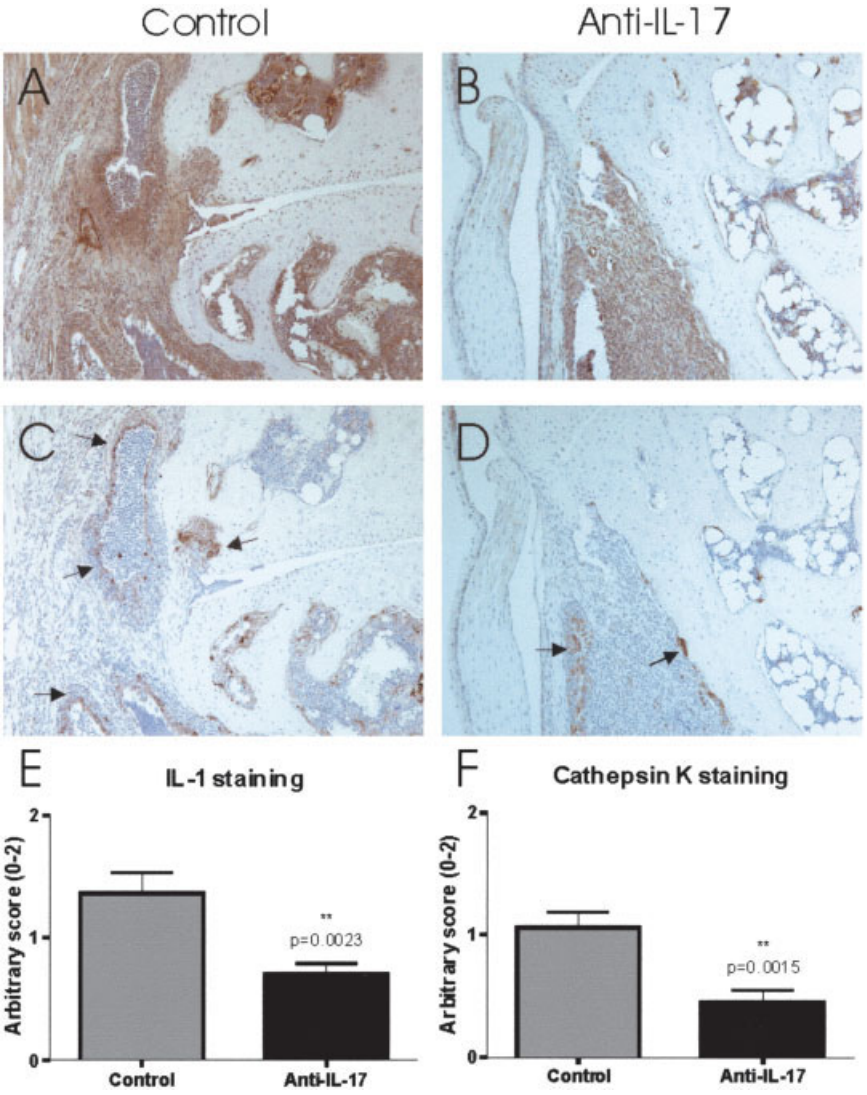

Figure 5. Suppression of interleukin-1 (IL-1) expression (**B** and **E**) and cathepsin K expression (**D** and **F**) by anti–IL-17 treatment in arthritic ankle joints of IL-1 receptor antagonist–deficient mice. Images in **A–D** show representative immunostaining results from a joint section in each group (original magnification × 200). Controls (**A** and **C**) were left untreated. **Arrows** in **C** and **D** indicate cathepsin K–positive osteoclast-like cells. Results in **E** and **F** are the mean and SEM arbitrary score of immunostaining for IL-1 (**E**) and cathepsin K (**F**) in at least 9 mice per group. ** = $P < 0.01$ versus control-treated group, by Student's *t*-test.

tigated the effect of blocking of IL-1 on the Th17 cell population. T cells were isolated from IL-1Ra$^{-/-}$ mice that were treated for 7 days with anti–IL-1 or control antibodies. FACS analysis of both the Th1 and the Th17 cell populations showed that anti–IL-1 treatment did not affect the levels of IFNγ+ Th1 cells (Figures 6A and B). However, the percentage of IL-17+ Th17 cells in the draining lymph nodes was significantly suppressed by neutralization of IL-1 (Figure 6C); a slight, but not significant, suppressive effect was also observed in the spleen (Figure 6D). These results support the position of IL-1 as the driving force behind the IL-17–producing Th17 cells in this IL-1Ra$^{-/-}$ murine arthritis model.

DISCUSSION

In this study we demonstrated that, in IL-1Ra$^{-/-}$ mice, TNFα does not contribute to the progression of

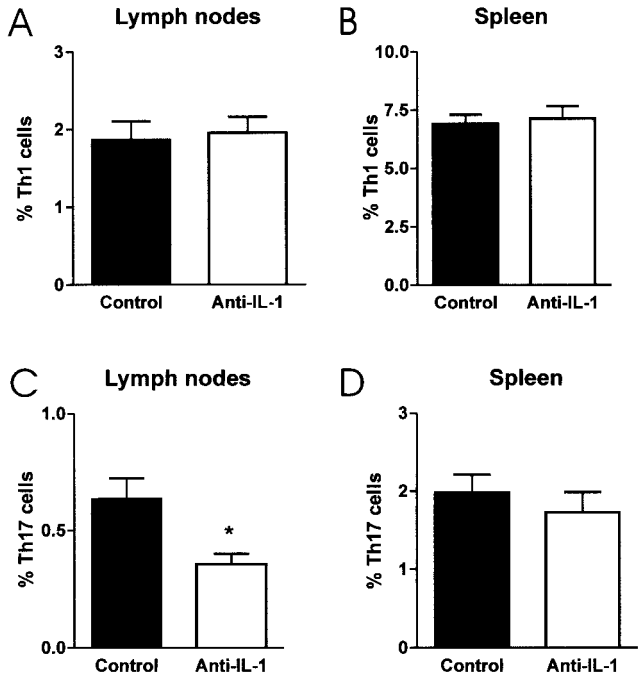

Figure 6. Fluorescence-activated cell sorter analysis of Th1 (**A** and **B**) and Th17 (**C** and **D**) cell subsets in the lymph nodes and spleens of interleukin-1 receptor antagonist–deficient (IL-1Ra$^{-/-}$) mice treated for 7 days with anti–IL-1 or control antibodies. The percentage of Th1 cells was not affected by blocking of IL-1, whereas the percentage of Th17 cells in the draining lymph nodes was significantly suppressed by anti–IL-1 treatment. Results are the mean and SEM percentage of Th1 or Th17 cells in 5–6 mice per group. $* = P < 0.05$ versus control-treated group, by Student's *t*-test.

IL-1–driven experimental arthritis. In addition, we showed that an increase in the number of Th17 cells already preceded the onset of arthritis, and high levels of IL-17 could be detected both locally and systemically. Our cytokine-blocking studies demonstrated that IL-17 contributes to the inflammation and bone erosion in this model, and the results suggest that IL-1 is the main driving force behind the IL-17–producing Th17 cells.

IL-1Ra$^{-/-}$ mice develop spontaneous arthritis as a result of excess IL-1 signaling due to the absence of the natural inhibitor IL-1Ra (5). Previously, it was shown that T cells are involved in this arthritic process, and that TNFα and IL-17 are important for the development of arthritis in this model (6,7). However, more interestingly, this animal model offers the opportunity to study the relative role of endogenous TNFα and IL-17 in the progression of an IL-1–driven experimental arthritis. During the development of arthritis in IL-1Ra$^{-/-}$ mice, the serum levels of IL-1, IL-17, and TNFα correlated with increasing age. Moreover, in the arthritic joint, increased mRNA levels of these cytokines were found, with a particularly impressive increase in the mRNA levels of IL-1 and IL-17.

Blocking of these cytokines after the onset of disease showed that, although TNFα expression was elevated in the arthritic joints, TNFα did not contribute significantly to the progression of arthritis. Blocking of TNFα did not reduce the macroscopic and histologic scores, even after raising the dose of TNFbp from 3 mg/kg to 10 mg/kg. These findings correspond with those obtained with anti-TNFα treatment during murine CIA, in which blocking of TNFα before or early after the onset of disease reduced the severity of the arthritis, but neutralization of TNFα in established CIA was not effective (33,34). In this IL-1–driven arthritis model in IL-1Ra$^{-/-}$ mice, the additional TNFα deficiency in the mice affects disease development, indicating that TNFα is important in the development of arthritis (6), but the results from blocking of TNFα after disease onset showed a lack of involvement of TNFα in the progression of arthritis. These data indicate that TNFα plays an important role in the early phase of arthritis, but that an established arthritis driven by IL-1 and involving T (and B) cells can become nonresponsive to anti-TNFα treatment.

Since T cells play an important role in this arthritis model (6), and stimulated splenic lymphocytes produce increased levels of IL-17 in IL-1Ra$^{-/-}$ mice (7), a role for IL-17–producing Th17 cells in the progression of arthritis was suspected. First, we demonstrated that IL-17 is highly up-regulated in the serum and synovium of arthritic IL-1Ra$^{-/-}$ mice. The main source of IL-17, formed by the Th17 cells, was clearly detectable in the draining lymph nodes and spleens of these mice. Remarkably, the increased percentage of Th17 cells even preceded the clinical onset of arthritis. Blocking of IL-17 resulted in a significant suppression of the macroscopic arthritis score, in comparison with the effects of control treatment. However, anti–IL-17 treatment only prevented further progression of arthritis and did not result in a decrease in the arthritis score. Apparently, the IL-1–driven arthritis model runs only partially through IL-17.

Histologic analysis showed that anti–IL-17 treatment significantly reduced inflammation and bone erosion, as compared with that in the control group, and a reduction in the local expression of cathepsin K and IL-1 accompanied this effect. This suggests that IL-17, initially driven by excess IL-1 production, contributes to the arthritic process by even further enhancing IL-1 levels in the arthritic joint, driving (part of) the local IL-1 pro-

duction and thereby acting both downstream and upstream of IL-1.

Neutralization of IL-1 almost completely reduced the macroscopic inflammation and was also very potent in reducing the histologic parameters of inflammation, cartilage destruction, and bone erosion. Even late after disease onset, in mice with macroscopic arthritis severity scores ≥1.5, anti–IL-1 treatment almost completely suppressed the arthritis in IL-1Ra$^{-/-}$ mice (results not shown).

Previously, IL-1 has been shown to play an essential role in T cell activation by regulating costimulatory molecules such as CD40 ligand and OX40 (35). Excess IL-1 in IL-1Ra$^{-/-}$ mice might therefore lead to enhanced T cell activation and, in combination with the enhanced IL-23 expression in IL-1Ra$^{-/-}$ mice (36), this enhanced T cell activation forms an ideal environment for Th17 cell formation. In our blocking study using anti–IL-1 monoclonal antibodies, this treatment significantly reduced the Th17 cell population in the draining lymph nodes. Moreover, stimulated splenic T lymphocytes in anti–IL-1–treated mice showed a reduction in IL-17 (results not shown). Similar responses were observed in IL-1Ra–treated mice during CIA (results not shown). This suggests that IL-1 contributes to the formation and/or activation of IL-17–producing T cells during arthritis, acting upstream of IL-17.

The effect of anti–IL-1 treatment was greater than expected, in terms of the role of T cells and IL-17+ T cells in this arthritis model. Although the initiation of the spontaneous arthritis in this model is undoubtedly driven by uncontrolled excess IL-1 production, the development of IL-17–producing T cells would potentially make this murine arthritis less dependent on IL-1. However, even after the onset of arthritis, IL-1 blocking led to almost complete remission, as assessed by macroscopic scoring of the ankle joints, and also resulted in an impressive reduction in inflammation, cartilage PG depletion, and bone erosion. This suggests that, apart from activated T cells and IL-17, other mediators drive the continuous IL-1 production in IL-1Ra$^{-/-}$ mice.

Results from recent studies suggest that Toll-like receptor 4 (TLR-4) and endogenous TLR ligands, including bacterial flora and damage-associated tissue components, might be involved in continuation of the arthritic process in IL-1Ra$^{-/-}$ mice (37). Endogenous TLR-4 ligands might play an important role in the progression of this arthritis. TLR-4 ligands have been found in RA synovial fluid, and Th17 cell numbers and IL-17 production are controlled by TLR-4 via the induction of IL-23 and IL-1 (37). This local cytokine environment involving IL-1 and IL-23 might explain the more pronounced inhibitory effects of anti–IL-1 treatment on the Th17 cell numbers in the draining lymph nodes compared with the spleens. High synovial IL-1 production in the arthritic IL-1Ra$^{-/-}$ joint promotes local Th17 cell differentiation and activation. Blocking of this local IL-1 production already affected the Th17 cells in the draining lymph nodes after 1 week of treatment, as shown in Figure 6C. Although the effects of anti–IL-1 on splenic Th17 cells were not significant, it is expected that prolonged blocking of IL-1 will also lead to significant suppression of Th17 cell numbers in the spleen.

Our findings in IL-1Ra$^{-/-}$ mice demonstrate that IL-1–driven experimental arthritis will not be blocked by neutralization of TNFα in later stages, during established disease, and that IL-17 produced by Th17 cells contributes to the progression of inflammation and destruction in this T cell–driven model. These data suggest that excess production of IL-1 during RA progression might result in nonresponsiveness to anti-TNFα treatment, and that IL-1–induced Th17 cell differentiation and activation will result in enhancement of IL-17 production, which contributes to increased inflammation and destruction of the joints.

AUTHOR CONTRIBUTIONS

Dr. Koenders had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Koenders, Devesa, Abdollahi-Roodsaz, Nicklin, Joosten.
Acquisition of data. Koenders, Devesa, Marijnissen, Boots, Walgreen, di Padova, Nicklin.
Analysis and interpretation of data. Koenders, Devesa, Boots.
Manuscript preparation. Koenders, Marijnissen, Abdollahi-Roodsaz, di Padova, Nicklin, Joosten, van den Berg.
Statistical analysis. Koenders, Devesa.

REFERENCES

1. Van den Berg WB. Is there a rationale for combined TNF and IL-1 blocking in arthritis? Clin Exp Rheumatol 2002;20(5 Suppl 27):S21–5.
2. Zwerina J, Redlich K, Schett G, Smolen JS. Pathogenesis of rheumatoid arthritis: targeting cytokines. Ann N Y Acad Sci 2005;1051:716–29.
3. Van der Laken CJ, Voskuyl AE, Roos JC, Stigter van Walsum M, de Groot ER, Wolbink G, et al. Imaging and serum analysis of immune complex formation of radiolabelled infliximab and anti-infliximab in responders and non-responders to therapy for rheumatoid arthritis. Ann Rheum Dis 2007;66:253–6.
4. Granowitz EV, Porat R, Mier JW, Pribble JP, Stiles DM, Bloedow DC, et al. Pharmacokinetics, safety and immunomodulatory effects of human recombinant interleukin-1 receptor antagonist in healthy humans. Cytokine 1992;4:353–60.
5. Horai R, Saijo S, Tanioka H, Nakae S, Sudo K, Okahara A, et al. Development of chronic inflammatory arthropathy resembling

rheumatoid arthritis in interleukin 1 receptor antagonist-deficient mice. J Exp Med 2000;191:313–20.
6. Horai R, Nakajima A, Habiro K, Kotani M, Nakae S, Matsuki T, et al. TNF-α is crucial for the development of autoimmune arthritis in IL-1 receptor antagonist-deficient mice. J Clin Invest 2004;114:1603–11.
7. Nakae S, Saijo S, Horai R, Sudo K, Mori S, Iwakura Y. IL-17 production from activated T cells is required for the spontaneous development of destructive arthritis in mice deficient in IL-1 receptor antagonist. Proc Natl Acad Sci U S A 2003;100:5986–90.
8. Chabaud M, Durand JM, Buchs N, Fossiez F, Page G, Frappart L, et al. Human interleukin-17: a T cell–derived proinflammatory cytokine produced by the rheumatoid synovium. Arthritis Rheum 1999;42:963–70.
9. Ziolkowska M, Koc A, Luszczykiewicz G, Ksiezopolska-Pietrzak K, Klimczak E, Chwalinska-Sadowska H, et al. High levels of IL-17 in rheumatoid arthritis patients: IL-15 triggers in vitro IL-17 production via cyclosporin A-sensitive mechanism. J Immunol 2000;164:2832–8.
10. Kotake S, Udagawa N, Takahashi N, Matsuzaki K, Itoh K, Ishiyama S, et al. IL-17 in synovial fluids from patients with rheumatoid arthritis is a potent stimulator of osteoclastogenesis. J Clin Invest 1999;103:1345–52.
11. Hwang SY, Kim HY. Expression of IL-17 homologs and their receptors in the synovial cells of rheumatoid arthritis patients. Mol Cells 2005;19:180–4.
12. Raza K, Falciani F, Curnow SJ, Ross EJ, Lee CY, Akbar AN, et al. Early rheumatoid arthritis is characterized by a distinct and transient synovial fluid cytokine profile of T cell and stromal cell origin. Arthritis Res Ther 2005;7:R784–95.
13. Harrington LE, Hatton RD, Mangan PR, Turner H, Murphy TL, Murphy KM, et al. Interleukin 17-producing $CD4^+$ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. Nat Immunol 2005;6:1123–32.
14. Park H, Li Z, Yang XO, Chang SH, Nurieva R, Wang YH, et al. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. Nat Immunol 2005;6:1133–41.
15. Kimura A, Naka T, Kishimoto T. IL-6-dependent and -independent pathways in the development of interleukin 17-producing T helper cells. Proc Natl Acad Sci U S A 2007;104:12099–104.
16. McGeachy MJ, Bak-Jensen KS, Chen Y, Tato CM, Blumenschein W, McClanahan T, et al. TGF-β and IL-6 drive the production of IL-17 and IL-10 by T cells and restrain T_H-17 cell-mediated pathology. Nat Immunol 2007;8:1390–7.
17. Kryczek I, Wei S, Vatan L, Escara-Wilke J, Szeliga W, Keller ET, et al. Cutting edge: opposite effects of IL-1 and IL-2 on the regulation of $IL\text{-}17^+$ T cell pool IL-1 subverts IL-2-mediated suppression. J Immunol 2007;179:1423–6.
18. Ivanov II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, Lafaille JJ, et al. The orphan nuclear receptor RORγt directs the differentiation program of proinflammatory $IL\text{-}17^+$ T helper cells. Cell 2006;126:1121–33.
19. Jovanovic DV, Di Battista JA, Martel-Pelletier J, Jolicoeur FC, He Y, Zhang M, et al. IL-17 stimulates the production and expression of proinflammatory cytokines, IL-β and TNF-α, by human macrophages. J Immunol 1998;160:3513–21.
20. Koenders MI, Lubberts E, van de Loo FA, Oppers-Walgreen B, van den Bersselaar L, Helsen MM, et al. Interleukin-17 acts independently of TNF-α under arthritic conditions. J Immunol 2006;176:6262–9.
21. Koenders MI, Lubberts E, Oppers-Walgreen B, van den Bersselaar L, Helsen MM, Kolls JK, et al. Induction of cartilage damage by overexpression of T cell interleukin-17A in experimental arthritis in mice deficient in interleukin-1. Arthritis Rheum 2005;52:975–83.
22. Fossiez F, Djossou O, Chomarat P, Flores-Romo L, Ait-Yahia S, Maat C, et al. T cell interleukin-17 induces stromal cells to produce proinflammatory and hematopoietic cytokines. J Exp Med 1996;183:2593–603.
23. Chabaud M, Miossec P. The combination of tumor necrosis factor α blockade with interleukin-1 and interleukin-17 blockade is more effective for controlling synovial inflammation and bone resorption in an ex vivo model. Arthritis Rheum 2001;44:1293–303.
24. Van Bezooijen RL, Van Der Wee-Pals L, Papapoulos SE, Lowik CW. Interleukin 17 synergises with tumour necrosis factor α to induce cartilage destruction in vitro. Ann Rheum Dis 2002;61:870–6.
25. Lubberts E, Joosten LA, Oppers B, van den Bersselaar L, Coenen-de Roo CJ, Kolls JK, et al. IL-1-independent role of IL-17 in synovial inflammation and joint destruction during collagen-induced arthritis. J Immunol 2001;167:1004–13.
26. Lubberts E, van den Bersselaar L, Oppers-Walgreen B, Schwarzenberger P, Coenen-de Roo CJ, Kolls JK, et al. IL-17 promotes bone erosion in murine collagen-induced arthritis through loss of the receptor activator of NF-κB ligand/osteoprotegerin balance. J Immunol 2003;170:2655–62.
27. Koenders MI, Kolls JK, Oppers-Walgreen B, van den Bersselaar L, Joosten LA, Schurr JR, et al. Interleukin-17 receptor deficiency results in impaired synovial expression of interleukin-1 and matrix metalloproteinases 3, 9, and 13 and prevents cartilage destruction during chronic reactivated streptococcal cell wall–induced arthritis. Arthritis Rheum 2005;52:3239–47.
28. Lubberts E, Schwarzenberger P, Huang W, Schurr JR, Peschon JJ, van den Berg WB, et al. Requirement of IL-17 receptor signaling in radiation-resistant cells in the joint for full progression of destructive synovitis. J Immunol 2005;175:3360–8.
29. Lubberts E, Koenders MI, Oppers-Walgreen B, van den Bersselaar L, Coenen-de Roo CJ, Joosten LA, et al. Treatment with a neutralizing anti-murine interleukin-17 antibody after the onset of collagen-induced arthritis reduces joint inflammation, cartilage destruction, and bone erosion. Arthritis Rheum 2004;50:650–9.
30. Koenders MI, Lubberts E, Oppers-Walgreen B, van den Bersselaar L, Helsen MM, Di Padova FE, et al. Blocking of interleukin-17 during reactivation of experimental arthritis prevents joint inflammation and bone erosion by decreasing RANKL and interleukin-1. Am J Pathol 2005;167:141–9.
31. Nicklin MJ, Hughes DE, Barton JL, Ure JM, Duff GW. Arterial inflammation in mice lacking the interleukin 1 receptor antagonist gene. J Exp Med 2000;191:303–12.
32. Kuiper S, Joosten LA, Bendele AM, Edwards CK III, Arntz OJ, Helsen MM, et al. Different roles of tumour necrosis factor α and interleukin 1 in murine streptococcal cell wall arthritis. Cytokine 1998;10:690–702.
33. Joosten LA, Helsen MM, Saxne T, van de Loo FA, Heinegard D, van den Berg WB. IL-1$\alpha\beta$ blockade prevents cartilage and bone destruction in murine type II collagen-induced arthritis, whereas TNF-α blockade only ameliorates joint inflammation. J Immunol 1999;163:5049–55.
34. Joosten LA, Helsen MM, van de Loo FA, van den Berg WB. Anticytokine treatment of established type II collagen–induced arthritis in DBA/1 mice: a comparative study using anti-TNFα, anti–IL-1α/β, and IL-1Ra. Arthritis Rheum 1996;39:797–809.
35. Nakae S, Asano M, Horai R, Sakaguchi N, Iwakura Y. IL-1 enhances T cell-dependent antibody production through induction of CD40 ligand and OX40 on T cells. J Immunol 2001;167:90–7.
36. Cho ML, Kang JW, Moon YM, Nam HJ, Jhun JY, Heo SB, et al. STAT3 and NF-κB signal pathway is required for IL-23-mediated IL-17 production in spontaneous arthritis animal model IL-1 receptor antagonist-deficient mice. J Immunol 2006;176:5652–61.
37. Abdollahi-Roodsaz S, Joosten LA, Koenders MI, Devesa I, Roelofs MF, Radstake TR, et al. Stimulation of TLR2 and TLR4 differentially skews the balance of T cells in a mouse model of arthritis. J Clin Invest 2008;118:205–16.