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Tumor Necrosis Factor–Interleukin-17 Interplay Induces S100A8, Interleukin-1β, and Matrix Metalloproteinases, and Drives Irreversible Cartilage Destruction In Murine Arthritis

Rationale for Combination Treatment During Arthritis

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Objective. **To examine whether synovial interleukin-17 (IL-17) expression promotes tumor necrosis factor (TNF)–induced joint pathologic processes in vivo, and to analyze the surplus ameliorative value of neutralizing IL-17 in addition to TNF during collagen-induced arthritis (CIA).**

Methods. **Adenoviral vectors were used to induce overexpression of IL-17 and/or TNF in murine knee joints. In addition, mice with CIA were treated, at different stages of arthritis, with soluble IL-17 receptor (sIL-17R), TNF binding protein (TNFBP), or the combination.**

Results. **Overexpression of IL-17 and TNF resulted in joint inflammation and bone erosion in murine knees. Interestingly, IL-17 strikingly enhanced both the joint-inflammatory and joint-destructive capacity of TNF. Further analysis revealed a strongly enhanced up-regulation of S100A8, IL-1β, and matrix metalloproteinase (MMP) messenger RNA, only when both TNF and IL-17 were present. Moreover, the increase in irreversible cartilage destruction was not merely the result of enhanced inflammation, but also was associated with a direct synergistic effect of these cytokines in the joint. S100A9 deficiency in mice protected against IL-17/TNF–induced expression of cartilage NITEGE neoepitopes. During established arthritis, the combination of sIL-17R and TNFBP was more effective than the anticytokine treatments alone, and significantly inhibited further joint inflammation and cartilage destruction.**

Conclusion. **Local synovial IL-17 expression enhances the role of TNF in joint destruction. Synergy between TNF and IL-17 in vivo results in striking exaggeration of cartilage erosion, in parallel with a synergistic up-regulation of S100A8, IL-1β, and erosive MMPs. Moreover, neutralizing IL-17 in addition to TNF further improves protection against joint damage and is still effective during late-stage CIA. Therefore, compared with anti-TNF alone, combination blocking of TNF and IL-17 may have additional therapeutic value for the treatment of destructive arthritis.**

Rheumatoid arthritis (RA) is a chronic disorder with unknown etiology. It is characterized by autoimmunity, infiltration of joint synovium by activated inflammatory cells, synovial hyperplasia, and progressive destruction of cartilage and bone. In the last decade,

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tumor necrosis factor (TNF) has been demonstrated to play a key role in the pathologic processes of RA (1–5). In the murine model of collagen-induced arthritis (CIA), it was shown that neutralizing TNF ameliorated CIA, especially when treatment was started before or early after the onset of disease (6). Importantly, neutralizing TNF in RA patients considerably improves joint inflammation and inhibits the progression of joint destruction (1–5). This led to use of the first anti-TNF biologic agents, for the treatment of RA.

Although anti-TNF therapy leads to considerable improvement of RA, this treatment does not stop progression of the disease in many patients. It is thus tempting to speculate that other cytokines are critical in inducing and maintaining the chronic destructive joint inflammation of RA, and therefore these other cytokines should be considered important additional targets for future therapy. In addition, further improvement of the current anti-TNF therapy to extend the beneficial effects to more RA patients is a challenge.

A recent study showed that anti-TNF treatment, despite ameliorating the arthritis, increased the production of interleukin-17 (IL-17) during CIA (7). IL-17 is a proinflammatory cytokine that is expressed in the synovium and synovial fluid of RA patients (8–11). Th17 cells have been identified as the main producers of this proinflammatory cytokine (12,13), but also γ/δ T cells, natural killer T cells, polymorphonuclear neutrophils, and mast cells have been shown to express or release IL-17. Studies have shown that IL-17 plays a role in many models of autoimmunity, including experimental arthritis (14,15). For instance, elevated levels of IL-17 were found in CIA, and neutralizing IL-17 at different stages of CIA ameliorated the severity of the arthritis (16,17). In addition, substantially diminished expression of CIA was observed in IL-17–deficient mice (18).

Furthermore, spontaneous arthritis development in IL-1 receptor antagonist–deficient mice was abolished by the inactivation of IL-17 expression, and blocking IL-17 after the onset of arthritis could prevent further aggravation of disease (19,20). In addition, using IL-17 receptor (IL-17R)–deficient mice, it was shown that IL-17R signaling plays a critical role in driving the synovial expression of proinflammatory and catabolic mediators during the development of chronic destructive arthritis (21). Interestingly, IL-17R signaling in radiation-resistant cells in the joint was required for full progression of destructive synovitis (22). These studies indicate a significant role for IL-17 in the development and progression of chronic destructive arthritis.

IL-17 is a potent inducer of various cytokines and chemokines (23–25). In addition, this T cell cytokine has been shown to have additive or even synergistic effects with TNF and IL-1 on cytokine induction and tissue destruction (26–30). Of note, an IL-1–independent role for IL-17 in synovial inflammation and joint destruction in CIA has been reported (17), whereas the bone-erosive effect of IL-17 in vivo is strongly mediated by RANKL (31). We previously demonstrated that IL-17 acts in a manner independent of TNF under arthritic conditions (32). In an ex vivo RA model utilizing cocultures of RA synovial tissue, a combination of TNF blockade with blockade of IL-1 and IL-17 was more effective than blockade of single cytokines for controlling synovial inflammation and bone resorption (33). However, the effect of in vivo blockade of IL-17 in combination with blockade of TNF on the pathologic processes of arthritis is still unknown.

In this study, we examined the additional pathologic contribution of synovial IL-17 in TNF-induced joint pathologic processes in vivo. In addition, we hypothesized that blocking IL-17 in addition to TNF should further improve the efficacy of anti-TNF therapy in CIA.

MATERIALS AND METHODS

Animals. Male DBA/1J mice were obtained from Janvier-Elevage. S100A9$^{-/-}$ mice, backcrossed to the C57BL/6 background for 10 generations, were provided by one of the authors (JR). Wild-type (WT) C57BL/6 mice (The Jackson Laboratory) were used as controls. All mice were used between 10 weeks and 12 weeks of age and were housed in filter-top cages under specific pathogen–free conditions. A standard diet and water were provided ad libitum. All animal procedures were approved by the institutional ethics committee.

Adenoviral vectors. Adenoviral TNF (AdTNF) and AdIL-17 were kindly provided by Dr. J. K. Kolls (Children's Hospital of Pittsburgh, Pittsburgh, PA). Viruses were under the regulation of a cytomegalovirus promoter and were constructed as reported previously (34). The recombinant adenoviruses contained fewer than 1 endotoxin unit/ml, as measured by the Limulus amebocyte lysate assay (BioWhittaker). A replication-deficient empty viral vector, AdDel70-3, was used as the control vector throughout the study.

Study protocol. Naive mice were anesthetized with isoflurane, and a small aperture in the skin of the knee was introduced for the intraarticular injection procedure. For local overexpression of IL-17 and/or TNF, 1×10^7 plaque-forming units (PFU) of each adenoviral vector in 6 ml phosphate buffered saline (PBS) was injected intraarticularly into the knee joint. As a control, the same amount of the control vector, AdDel70-3, was used. Four or 10 days thereafter, mice were killed by cervical dislocation, and the knee joints were isolated for histology.

Histology. For standard histology, 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 mm in size, were mounted on SuperFrost slides (Menzel-Gläser). Hematoxylin and eosin staining and Safranin O staining were performed to study the joint pathologic features. The severity of arthritis in the joints was scored on a scale of 0–3 (where 0 = no pathology and 3 = maximal cellularity), as previously described (17), for 5 different parameters: joint inflammation, proteoglycan (PG) depletion from the cartilage matrix, chondrocyte death, cartilage surface erosion, and bone erosion. Histopathologic changes were scored on 5 semiserial sections, spaced 140 mm apart. Scoring was performed in a blinded manner by 2 independent observers.

VDIPEN neoepitope staining. Irreversible PG damage by matrix metalloproteinase (MMP) activity in the cartilage was assessed using immunohistochemical detection of the VDIPEN neoepitope, as previously described (35). Briefly, joint sections were deparaffinized, rehydrated, and digested in chondroitinase to remove chondroitin sulfate from the PGs. Sections were treated with 1% H_2O_2 in methanol, followed by treatment with 0.1% Triton X-100. After incubation with 1.5% normal goat serum, sections were incubated overnight at 4°C with rabbit anti-VDIPEN IgG (a kind gift from Dr. John Mort, Montreal, Quebec, Canada) or normal rabbit IgG.

RNA isolation and quantitative polymerase chain reaction (PCR) analysis. Mice were killed by cervical dislocation, immediately followed by dissection of the patella with adjacent synovium. RNA from the patellar cartilage and pooled synovial biopsy tissue was isolated, as described previously (35). Real-time quantitative PCR was performed using the ABI Prism 7000 Sequence Detection System, for quantification with SYBR Green Master Mix and melting curve analysis. Relative quantification of the PCR signals was performed by comparing the threshold cycle (C_t) value, in duplicate, for the gene of interest of each sample with the C_t values for the reference gene, GAPDH. The quantitative PCR analysis for each sample was performed in duplicate.

NF-κB luciferase bioassay. The ability of IL-17 and TNF to activate NF-κB was tested on responsive fibroblast NF-κB reporter cells. One day prior to conducting the experiment, NIH-3T3 reporter cells were seeded in Krystal 2000 96-well plates (Thermo Labsystems) at a concentration of $2–4 \times 10^4$ cells/well. Cells were cultured in 5% CO_2 at 37°C. Six hours after the addition of IL-17, TNF, or the combination (both at 10 ng/ml), NF-κB activation was measured through determination of intracellular luciferase production. Luciferase activity was quantified using the Bright-Glo luciferase assay system (Promega), followed by luminometric detection, according to the manufacturer's protocol (Polarstar Galaxy; BMG).

Measurement of NITEGE neoepitopes in patellar cartilage. Patellae were derived from S100A9$^{-/-}$ mice and WT mice, and the synovium was carefully removed. Patellae were incubated for 24 hours with 100 ng/ml of IL-17, TNF, IL-17 plus TNF, or IL-1β as a positive control (all recombinant murine cytokines from R&D Systems). Patellae were decalcified in EDTA, and 5-μm sections were stained with anti-NITEGE antibodies, a kind gift from Dr. John Mort, and counterstained with hematoxylin (Merck).

Induction of CIA. Bovine type II collagen (CII) was prepared as described previously (6). CII was diluted in 0.05*M* acetic acid to a concentration of 2 mg/ml and emulsified in equal volumes of Freund's complete adjuvant (2 mg/ml of *Mycobacterium tuberculosis* strain H37Ra; both from Difco). The DBA/1J mice were immunized intradermally at the base of the tail with 100 μg of CII. On day 21, mice received an intraperitoneal (IP) booster injection of 100 μg of CII dissolved in PBS, and the onset of arthritis usually occurred a few days after the booster injection. Mice were carefully examined 3 times per week for the visual appearance of arthritis in the peripheral joints, and scores for disease activity were given as previously described (36). The clinical severity of arthritis (arthritis score) was graded on a scale of 0–2 for each paw, according to changes in redness and swelling in the digits or in other parts of the paws.

Anticytokine treatment. Recombinant murine soluble IL-17R:Fc fusion protein (sIL-17R) (37) was kindly provided by Dr. S. D. Lyman (Immunex, Seattle, WA). To neutralize TNF, mice were treated with dimerically linked PEGylated soluble p55 TNF receptor I (Amgen). This so-called TNF binding protein (TNFBP) showed efficacy in murine streptococcal cell wall (SCW)–induced arthritis (38) and CIA (39). Mice were injected IP with 3 mg/kg sIL-17R, TNFBP, or the combination. Bovine serum albumin (BSA; Sigma-Aldrich), at a dose of 6 mg/kg, was used as a control. A total of 4 injections were given on alternate days. Anticytokine treatment was started before the clinical onset of disease (day 25) or during established arthritis (macroscopic arthritis scores of 2.0–2.5, on a scale of 0–8).

Statistical analysis. Differences between experimental groups were tested using one-way analysis of variance or the Kruskal-Wallis test with Dunn's test for multiple comparisons, unless stated otherwise. *P* values less than 0.05 were considered significant. Results are expressed as the mean ± SEM.

RESULTS

Enhancement of the inflammatory and joint-destructive capacity of TNF by IL-17. To investigate the individual potency of IL-17 and TNF, as well as the combination of these 2 cytokines, in the induction of joint pathologic processes, naive mice were intraarticularly injected with an adenoviral vector expressing IL-17 and/or TNF, or a control vector, directly into the knee joint. The adenoviruses were injected at a concentration of 1×10^7 PFU in 6 μl, resulting in rapid overexpression of the cytokines in the first 24 hours (mean ± SEM peak expression in synovial washouts reaching 544 ± 73 pg/ml for IL-17 and 589 ± 208 pg/ml for TNFα), followed by a fast decline from these levels and loss of expression within 1 week.

Four and 10 days after injection, the knee joints were isolated for histology. Overexpression of IL-17 resulted in influx of inflammatory cells into the joint, accompanied by mild bone erosion (Figures 1 and 2A). Local TNF gene transfer resulted in slightly more joint inflammation as well as a higher degree of cartilage PG

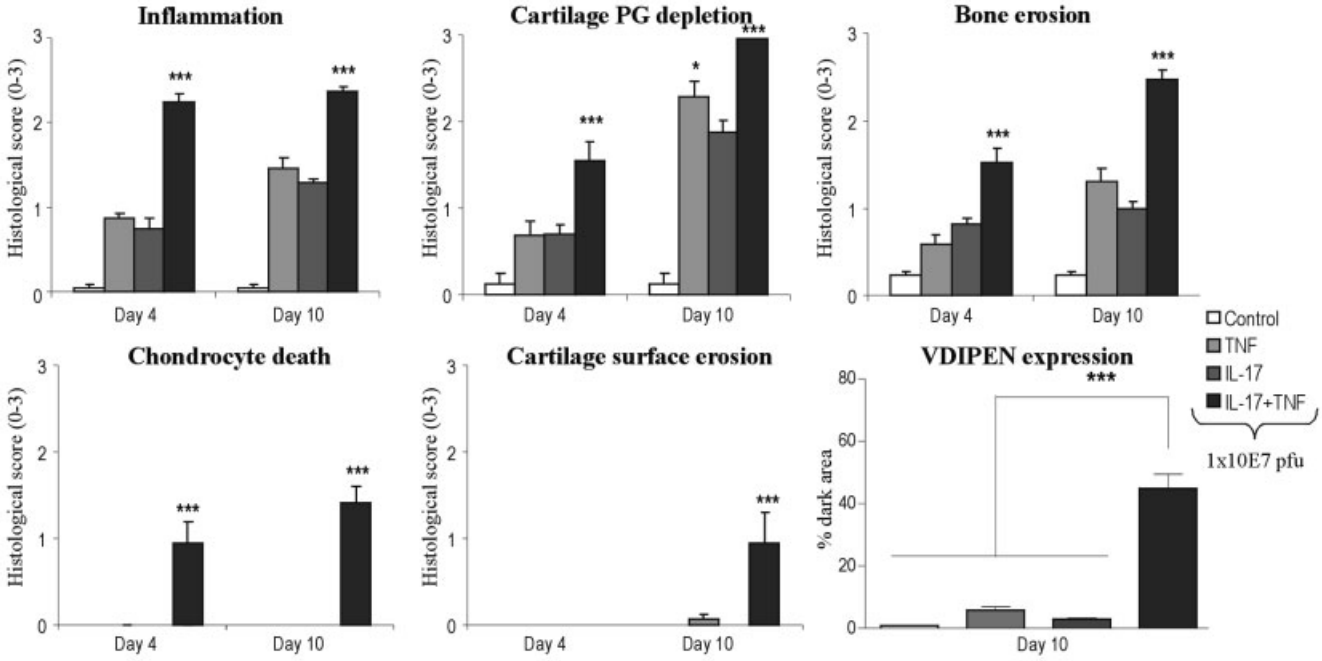

Figure 1. Results of histologic analysis and VDIPEN staining to determine irreversible cartilage destruction in murine knee joints. Histologic scores were determined for the extent of inflammatory infiltrate, cartilage proteoglycan (PG) depletion, bone erosion, chondrocyte death, cartilage surface erosion, and VDIPEN staining after local overexpression of interleukin-17 (IL-17) and/or tumor necrosis factor (TNF) in the knee joints of naive mice. Adenoviruses (AdControl, AdIL-17, AdTNF, or the combination of AdIL17 plus AdTNF at 1×10^7 plaque-forming units [PFU]) were injected intraarticularly, and on days 4 and 10, the knee joints were isolated for analysis of histologic parameters and VDIPEN expression. Results are the mean ± SEM of 4–6 mice per group. $* = P < 0.05$; $*** = P < 0.001$ versus AdControl.

depletion than that following gene transfer with IL-17 (Figures 1 and 2A). Of note, although overexpression of both proinflammatory cytokines led to cartilage PG depletion, hardly any irreversible cartilage damage

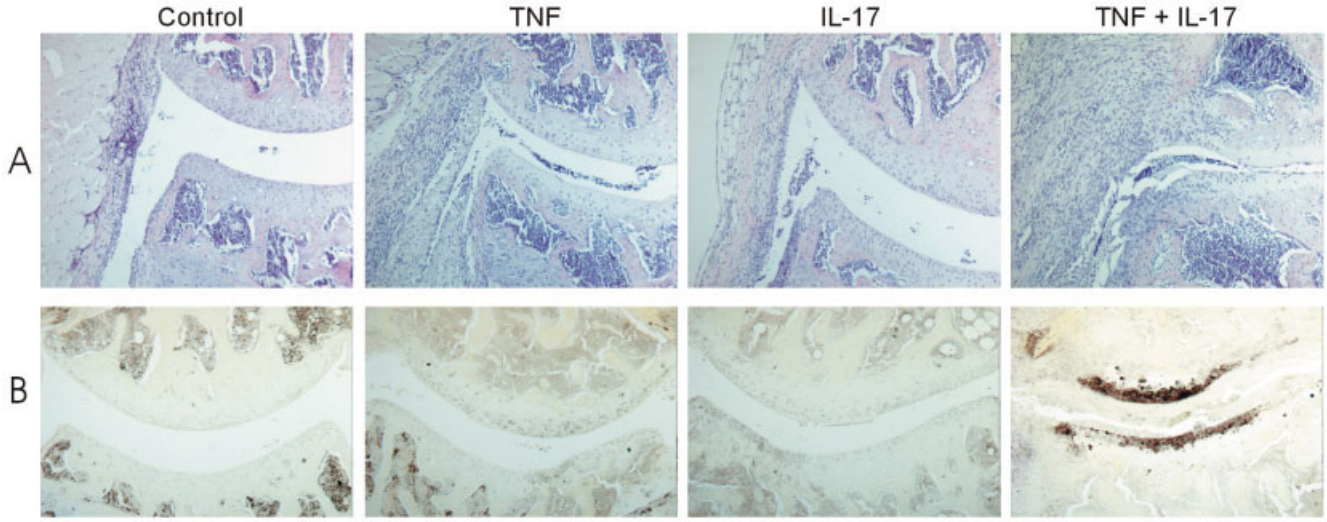

Figure 2. IL-17 enhances the joint-destructive capacity of TNF. Inflamed knee joints were stained with hematoxylin and eosin for histologic features **(A)** or stained for VDIPEN expression as a marker for matrix metalloproteinase–mediated cartilage destruction **(B)**, at 10 days after a single injection of an adenoviral vector expressing IL-17, TNF, the combination of IL-17 plus TNF, or a control vector. Note the enhanced joint inflammation and joint destruction in the IL-17/TNF combination group. Original magnification × 100. See Figure 1 for definitions.

(chondrocyte death and surface erosions) was found in these groups; only TNF overexpression induced some cartilage erosion on day 10 (Figure 1).

The combination of IL-17 and TNF enhanced joint inflammation and bone erosion, in an additive manner (Figures 1 and 2A). Intriguingly, although IL-17 itself did not induce irreversible cartilage destruction, IL-17 in combination with TNF synergistically enhanced chondrocyte death and cartilage surface erosions (Figures 1 and 2A). VDIPEN, a metalloproteinase-generated aggrecan neoepitope, was hardly detectable in the knee joints of mice injected with only AdTNF or only AdIL-17. However, the amount of VDIPEN expression increased dramatically when TNF and IL-17 were simultaneously overexpressed in the joint (Figures A and 2B). These findings show that the presence of both TNF and IL-17 amplifies the joint-inflammatory and joint-destructive capacity of the single cytokines.

Synergistic up-regulation of S100A8 and IL-1β by TNF and IL-17. Synovial biopsy tissue and isolated cartilage layers from the TNF/IL-17 overexpression study were further analyzed to detect the gene regulation that accompanies aggravated joint inflammation and, more importantly, the synergistic effects on cartilage destruction. Quantitative PCR analysis demonstrated that, when compared to the control group, both TNF and IL-17 induced a $\pm$60-fold up-regulation of IL-1β messenger RNA (mRNA), and even induced a $\pm$350-fold increase in S100A8 mRNA, an alarmin that was recently found to regulate severe cartilage destruction (40) (Figure 3A). Interestingly, an even stronger, more additive up-regulation of S100A8 and IL-1β was found in the synovium of mice overexpressing both TNF and IL-17 (Figure 3A). In contrast, cartilage-destructive MMP-14 and bone-protective osteoprotegerin mRNA were slightly up-regulated, to a similar extent, in all 3 cytokine groups. The levels of mRNA for the osteoclast regulator RANKL were more potently up-regulated by IL-17 than by TNF, while the combination of IL-17 plus TNF did not further enhance the expression of RANKL mRNA (Figure 3A).

TNF and IL-17 synergy associated with enhanced activation of NF-κB and up-regulation of MMPs. To study the effect of combined stimulation with TNF and IL-17 on intracellular NF-κB signaling, we used an NF-κB reporter fibroblast that produces luciferase upon NF-κB activation. Following stimulation of the cells with equal concentrations of TNF and IL-17, we observed that both cytokines were able to activate NF-κB, with TNF being more potent than IL-17. The combined stimulation with TNF and IL-17 resulted in a significantly enhanced NF-κB activation (Figure 3C).

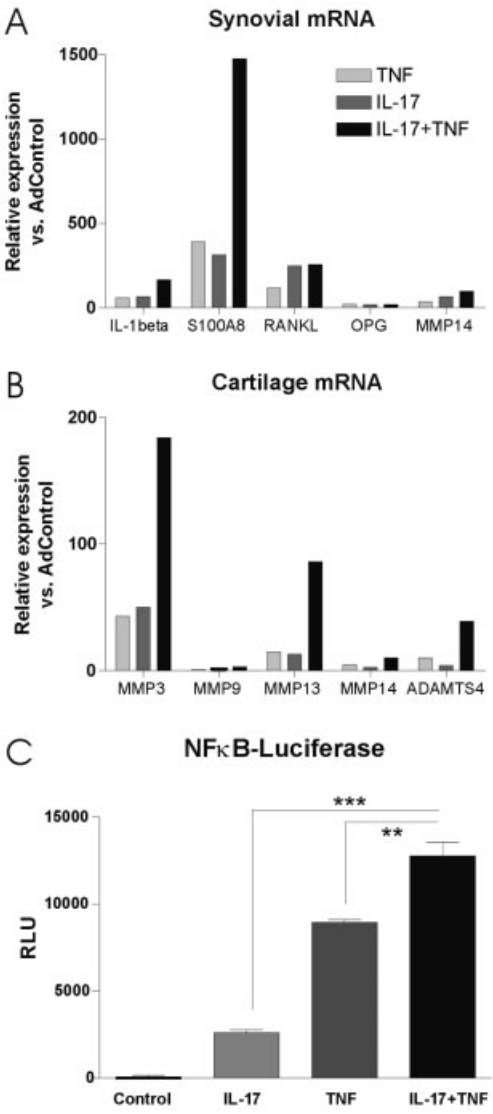

Figure 3. Effects of overexpression of TNF and/or IL-17 on gene regulation and NF-κB activation during aggravated inflammation in murine joints. **A** and **B,** Combined overexpression of TNF and IL-17 resulted in enhanced up-regulation of synovial S100A8 and IL-1β mRNA, with little effect on synovial RANKL, osteoprotegerin (OPG), or matrix metalloproteinase 14 (MMP-14) mRNA expression (**A**), and enhanced up-regulation of cartilage MMP-3 and MMP-13 mRNA, with little effect on cartilage MMP-9, MMP-14, or ADAMTS-4 mRNA expression (**B**). Adenoviruses (AdControl, AdIL-17, AdTNF, or the combination of AdIL17 plus AdTNF) were injected intraarticularly, and on day 4, synovial biopsy tissue and patellar cartilage were isolated for quantitative polymerase chain reaction analysis. Representative results from 1 of 2 independent in vivo experiments are shown. Values are the mean of 2 pooled samples from 3 mice per group. **C,** Stimulation of a fibroblast luciferase reporter cell line with the combination of TNF and IL-17 resulted in clear additive effects on NF-κB activation, as compared with either cytokine alone. Bars show the mean $\pm$ SEM relative light units (RLU) of luciferase activity. ** = $P < 0.01$; *** = $P < 0.001$, by one-way analysis of variance. See Figure 1 for other definitions.

The association of the TNF/IL-17 interplay with regulation of target genes was shown not only in synovial tissue, but also in cartilage. Following overexpression of TNF or IL-17 in cartilage chondrocytes, the mRNA expression of various MMPs was up-regulated compared to that in the control group (Figure 3B). However, the chondrocytes showed a much stronger up-regulation of MMP-3, MMP-13, and ADAMTS-4 when both cytokines were present (Figure 3B). For example, the expression of MMP-13 was enhanced by ±14-fold by AdTNF or AdIL-17 compared to that in the control group, and when AdTNF and AdIL-17 were combined, this expression showed a further increase of ±6-fold compared to that with the single cytokines.

Direct synergistic effects of IL-17 and TNF on cartilage destruction. After injection of 1×10^7 PFU of each cytokine virus, we demonstrated that only the combination of IL-17 and TNF induced severe cartilage destruction. In the next experiment, we aimed to demonstrate that TNF and IL-17 act in synergy directly on cartilage destruction, and sought to exclude the possibility that the enhancements in MMP expression, VDIPEN staining, and cartilage destruction were caused only by increased inflammation. In this experiment, lower doses of AdIL-17/AdTNF were used in the combination group (each 3×10^6 PFU), which resulted in a comparable level of synovial inflammation as that with the normal dose of each single-cytokine virus (kept at 1×10^7 PFU). As shown in Figure 4, the combination of IL-17 and TNF at this lower dose no longer resulted in aggravated PG depletion or bone erosion. Whereas the degree of inflammation appeared to be coupled to bone erosion and showed the same relative decline after reducing the viral dose, parameters of cartilage destruction were more strongly affected by reducing the viral dose. Interestingly, histologic analysis of the cartilage demonstrated that, again, only the combination of IL-17 and TNF resulted in irreversible chondrocyte death, cartilage erosion, and MMP-mediated VDIPEN expression (Figure 4), suggesting that the synergy between TNF and IL-17 induces cartilage destruction independent of the degree of inflammation.

Protective effects of S100A9 deficiency against IL-17/TNF–induced cartilage damage in vitro. Our overexpression studies in vivo demonstrated that IL-17 in the presence of TNF induces irreversible cartilage destruc-

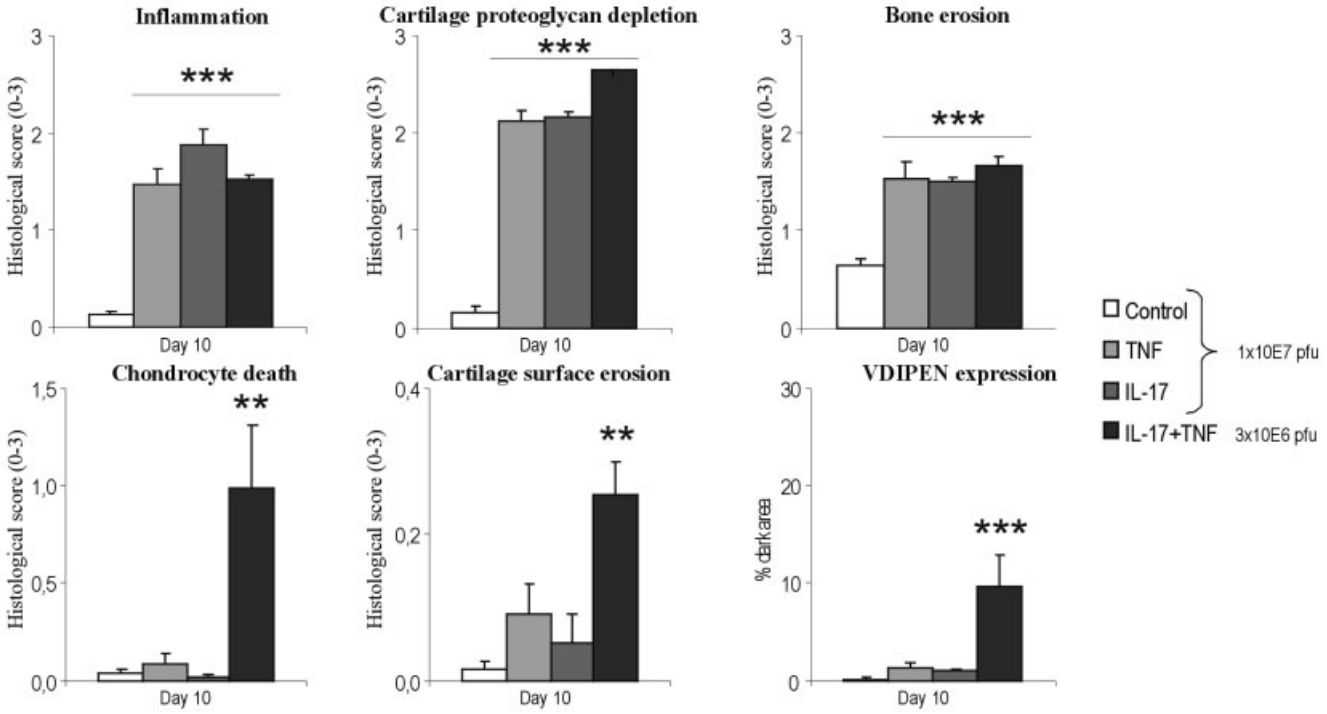

Figure 4. After reducing the virus dose for the combination group (IL-17 plus TNF), to reach comparable levels of synovial inflammation among the 3 cytokine treatment groups, only the combination of TNF and IL-17 resulted in irreversible cartilage destruction in murine knee joints. Adenoviruses (AdControl, AdIL-17, AdTNF, or the combination of AdIL17 plus AdTNF) were injected intraarticularly, and on day 10, the knee joints were isolated for analysis of histologic parameters and VDIPEN expression. Results are the mean ± SEM of 6 mice per group. ** = $P < 0.01$; *** = $P < 0.001$ versus AdControl. See Figure 1 for definitions.

tion, associated with enhanced mRNA expression of S100A8, MMPs, and ADAMTS. Previous studies demonstrated that S100A8 is an important inducer and activator of MMPs and aggrecanases (40). To determine whether the up-regulation of S100A8 by IL-17/TNF is indeed responsible for the cartilage-destructive effect, we performed an in vitro study with cartilage of S100A9-deficient mice. Patellar cartilage was isolated from WT and S100A9-deficient mice, the latter of which express neither S100A9 nor S100A8, probably because of the high turnover of free S100A8 in the absence of its binding partner, S100A9. In vitro stimulation of the WT cartilage with IL-17 plus TNF induced clear NITEGE neoepitope expression as a marker for aggrecanase-mediated cartilage destruction (Figure 5C), to an extent comparable with that in cartilage stimulated with IL-1β as a positive control (Figure 5B), whereas no NITEGE neoepitope expression was observed in unstimulated mouse patellae (Figure 5A). However, induction of NITEGE neoepitopes by IL-17 and TNF was almost completely prevented in cartilage derived from S100A9-deficient mice (Figure 5D), demonstrating an important role for S100A8/S100A9 proteins in this process of IL-17/TNF–mediated cartilage destruction.

Reduction in the severity of arthritis after blockade of IL-17 and TNF during CIA. To examine the effect of individual blocking as well as combination blocking of TNF and IL-17 during experimental arthritis, DBA/1J mice were immunized with CII in emulsified Freund's complete adjuvant. Before the visible onset of arthritis or during established arthritis, mice were systemically treated on alternate days with a total of 4 injections of 3 mg/kg sIL-17R, TNFBP, the combination of sIL-17R plus TNFBP, or BSA as a control.

Neutralizing TNF before the onset of CIA significantly suppressed the severity of the disease, as shown by a significant decrease in the macroscopic arthritis score in the TNFBP-treated group (Figure 6A). Nevertheless, mice that were treated with the combination of sIL-17R and TNFBP showed even greater reductions in the severity of arthritis (Figure 6A), suggesting that neutralization of IL-17 in addition to TNF has further therapeutic efficacy in CIA.

In addition to cytokine blocking before arthritis

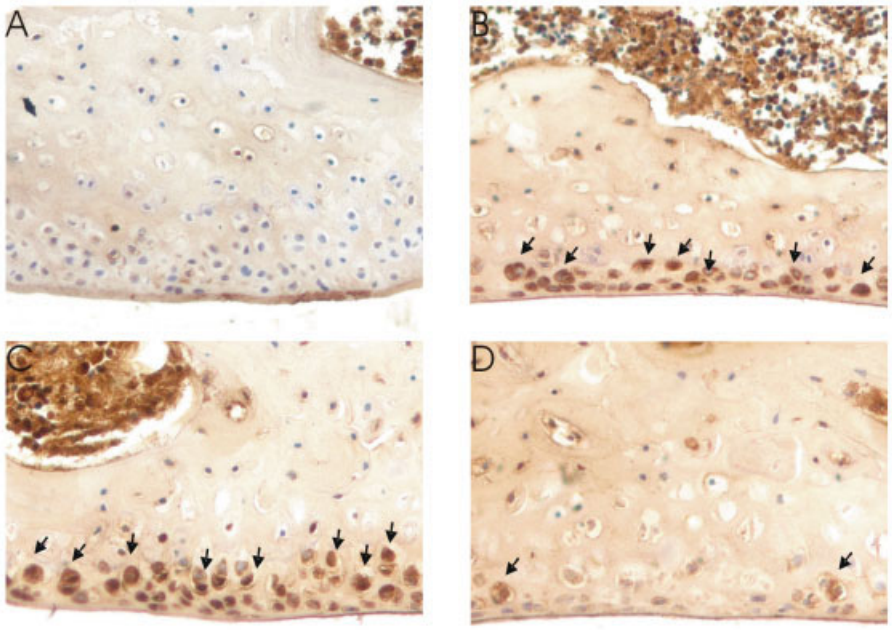

Figure 5. NITEGE neoepitope expression, a measure of aggrecanase-mediated cartilage destruction, was determined by NITEGE antibody staining of unstimulated wild-type mouse patellae (control) **(A)**, wild-type mouse patellae stimulated with 100 ng of IL-1β **(B)**, or wild-type mouse patellae stimulated with the combination of IL-17 plus TNF **(C)** for 24 hours. IL-17 plus TNF elevated the expression of NITEGE neoepitopes to levels comparable with those in the positive control IL-1β–stimulated group, and this IL-17/TNF–induced NITEGE expression was almost completely prevented in S100A9$^{-/-}$ mouse patellae **(D)**. **Arrows** indicate clear brown staining for NITEGE neoepitopes around chondrocytes. See Figure 1 for definitions.

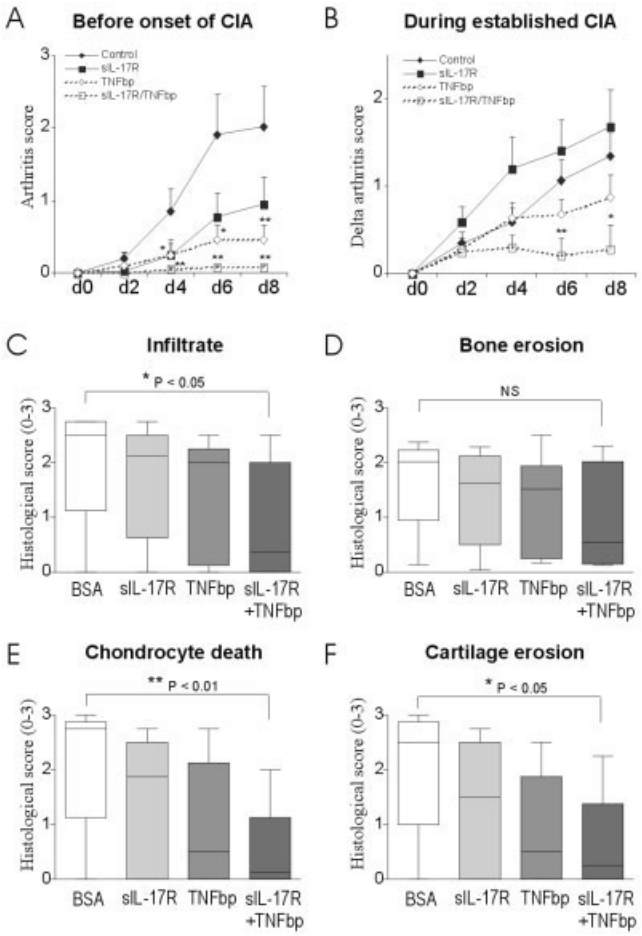

Figure 6. Effects of neutralizing endogenous IL-17 and/or TNF on murine collagen-induced arthritis (CIA). **A** and **B,** Neutralizing endogenous IL-17 and/or TNF before disease onset **(A)** or during established CIA **(B)** reduced disease severity. DBA/1J mice were immunized with type II collagen, and a booster injection was given on day 21. Mice were treated with intraperitoneal injections of 3 mg/kg soluble IL-17 receptor (sIL-17R), TNF binding protein (TNFBP), or the combination. Bovine serum albumin (BSA) was used as a control. A total of 4 injections on alternate days were given. The appearance of arthritis was assessed, and the severity of arthritis was visually scored on a scale of 0–2 for each paw. Treatment was started just before the expected onset (day 25) **(A)** or when an arthritis score of 2.0–2.5 had been reached **(B)**. **C–F,** The histologic parameters of influx of inflammatory infiltrates **(C)**, bone erosion **(D)**, chondrocyte death **(E)**, and cartilage erosion **(F)** were scored on day 8. Results in **A** and **B** are the mean ± SEM arthritis score for 10 mice per group. Results in **C–F** are box plots, where the boxes represent the 25th to 75th percentiles, the lines within the boxes represent the median, and the lines outside the boxes represent the 10th and 90th percentiles for 10 mice per group. NS = not significant (see Figure 1 for other definitions).

onset, equal doses of sIL-17R and TNFBP were used in the treatment of established CIA. Treatment was started in mice when an arthritis score of 2.0–2.5 had been reached, using the same protocol of 4 IP injections of sIL-17R, TNFBP, the combination, or BSA as a control on alternate days. In this late stage of CIA, blocking of IL-17 or TNF alone did not significantly reduce the progression of CIA (Figure 6B). Interestingly, however, neutralizing the combination of both IL-17 and TNF almost completely inhibited further progression of disease (Figure 6B).

Histologic analysis revealed that there was a significant suppression of not only inflammation but also cartilage destruction when the treatment combination of sIL-17R and TNFBP was used (Figures 6C–F). Bone erosion in the sIL-17R/TNFBP treatment group of arthritic mice was not significantly reduced (Figure 6D). Although sIL-17R or TNFBP alone did not have any significant effects on the extent of inflammatory infiltrate, chondrocyte death, and cartilage surface erosion, the combination of TNFBP plus sIL-17R was clearly effective in preventing cartilage destruction and protecting its repair capacity (Figures 6E and F).

DISCUSSION

In the present study, we demonstrated that the presence of IL-17 in the joint enhanced the progression of joint destruction induced by local TNF. Moreover, neutralizing IL-17 in addition to TNF during different stages of CIA further ameliorated the severity of arthritis and inhibited progression of CIA. In particular, TNF and IL-17 were demonstrated to synergistically enhance cartilage destruction independent of the degree of inflammation. This implies that combined anti–IL-17/anti-TNF therapy may have additional therapeutic value for the treatment of destructive arthritis, compared with anti-TNF alone.

Anti-TNF therapy in RA patients leads to considerable improvement of arthritis (1–5). However, not all patients respond to anti-TNF therapy. The cytokine cascades involved in joint inflammation are very complicated, and many interactions may take place, leading to a complex communication network between different cell types. Therefore, more knowledge is needed to unravel the critical interactions between key players in the arthritis process to further improve current therapy. In the last couple of years, ample data have confirmed the beneficial effects of neutralizing IL-17, as shown in ex vivo studies using human material from patients with RA as well as in experimental arthritis models. It has also become clear that IL-17 can fine-tune the arthritis process by inducing and working together with different

cytokines and chemokines in an additive or synergistic way (15,41).

In this study, we combined blocking of TNF and IL-17 to enhance therapeutic efficacy. The rationale for using this combination is as follows. TNF is produced by different cell types, including T cells. Activated macrophages may be the predominant cell type responsible for TNF production during arthritis. IL-17 expression is restricted and is predominantly produced by activated Th17 cells. Since T cells play an important role in exacerbations of (destructive) arthritis and during flares, IL-17 may be the major driving cytokine in this process (16,42). In addition, we previously showed that IL-17 has activities in vivo that are either IL-1– and TNF-independent or IL-1– and TNF-dependent (16,17,32,35). Therefore, blocking both TNF and IL-17 may control both the macrophage as well as T cell activity pathways in the arthritis process.

Interestingly, a recent study demonstrated that anti-TNF treatment in CIA might cause an expansion of IL-17–producing T cells (7), suggesting that blocking IL-17 in addition to TNF might have enhanced therapeutic effect. Our data from the present study support the hypothesis of effective combination therapy by showing an increased beneficial response in CIA after neutralizing both TNF and IL-17.

Cartilage surface erosion is due to a loss of collagen structure, mediated by specific enzymes such as MMPs. Immune complexes play an important role in this type of destruction. Overexpression of TNF or IL-17 alone did not result in loss of cartilage structures, and this may be due to the lack of immune complexes formed during IL-17– and TNF-induced joint inflammation in normal mice. In contrast, overexpression of both IL-17 and TNF in the knee joints of normal mice did result in cartilage surface erosion. This implies that overexpression of IL-17 and TNF together is able to induce a process responsible for the induction of cartilage destruction.

Quantitative PCR analysis revealed that the combination of TNF and IL-17 induced a synergistic up-regulation of MMP-3 and MMP-13, which might well explain the MMP-mediated VDIPEN expression in the knee joints, as revealed with immunohistochemistry. In addition, S100A8 was tremendously up-regulated, especially by the combination of these 2 inflammatory cytokines. This enhanced gene regulation was accompanied by enhanced NF-kB activation, as demonstrated with a luciferase reporter fibroblast assay. Other intracellular mechanisms, via the transcription factor CCAAT/enhancer binding protein, or enhanced mRNA stability might also contribute to the TNF/IL-17 synergy, as previously described (43–45).

Previous studies from our group have shown that S100A8 can stimulate chondrocytes to produce MMPs, to activate latent MMPs, and to degrade the actual cartilage matrix, and these effects were even further enhanced in the presence of IL-1 (46). These previous findings and our current observations in S100A9$^{-/-}$ mice lacking IL-17/TNF–induced NITEGE epitopes suggest that the enhanced up-regulation of S100A8 may very well contribute to the cartilage destruction induced by TNF/IL-17 synergy.

One of the difficulties associated with anti-cytokine therapy in arthritis is that after cessation of treatment, arthritis relapses. Indeed, we found that 7 days after the last sIL-17R or TNFBP injection, treated mice with CIA no longer showed a significant difference in the arthritis score compared with untreated mice with CIA. In contrast, neutralizing both IL-17 and TNF still resulted in significantly less disease expression 1 week after cessation of treatment, although the combination treatment was not able to switch off CIA (results not shown). This suggests that IL-17/TNF combination therapy is able to slow down the process of upcoming arthritis after cessation of therapy. However, it also indicates that continuous blocking of IL-17/TNF will be needed to keep the suppression of CIA ongoing.

TNF is hardly detectable in the later stages of CIA, and previous blocking studies with anti–IL-1 and anti-TNF, performed in our laboratory, have shown that TNF plays an important role in early CIA, whereas IL-1 is important in early and established CIA (6). The results of the present study are consistent with the notion of an early beneficial effect of anti-TNF therapy.

Of note, in the murine model of SCW-induced arthritis, it was found that TNF is a principal cytokine contributing to the joint swelling, since TNF-deficient mice showed hardly any increase in joint thickness (47). In these TNF-deficient mice, local injection of SCW fragments into the knee joint resulted in local synovial expression of IL-1, indicating that synovial IL-1 production could be induced in a TNF-independent manner (35). Recently, we showed that IL-17 is a potent inducer of IL-1 in CIA (18), and in another study using the murine SCW-induced arthritis, IL-17R signaling was critical for synovial IL-1 expression in the late chronic destructive phase of arthritis (21). This suggests that IL-17 is an upstream mediator of synovial IL-1. Our overexpression study showed that IL-1 was one of the genes that were synergistically up-regulated by TNF and IL-17. Therefore, we hypothesized that neutralizing

IL-17 in addition to TNF may inhibit synovial IL-1 expression, which is of additional benefit in the suppression of CIA. Studies are in progress to unravel the truth of this hypothesis.

In conclusion, this study showed that neutralizing IL-17 in addition to TNF was still effective in the late stages of CIA and was still able to suppress the progression of CIA under conditions in which blocking of IL-17 or TNF alone had not been effective. Therefore, it will be interesting to consider this potential new therapy for the treatment of human RA, especially in RA patients whose condition has not responded to anti-TNF therapy alone.

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All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. 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 conception and design. Koenders, Devesa, Lubberts, Joosten, van Lent, van de Loo, van den Berg.

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