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*J Immunol* 1999; 163:5049-5055; ; 
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IL-1αβ Blockade Prevents Cartilage and Bone Destruction in Murine Type II Collagen-Induced Arthritis, Whereas TNF-α Blockade Only Ameliorates Joint Inflammation

Leo A. B. Joosten,2* Monique M. A. Helsen,* Tore Saxne,†† Fons A. J. van de Loo,* Dick Heinegård,* and Wim B. van den Berg*

Anti-TNF-α treatment of rheumatoid arthritis patients markedly suppresses inflammatory disease activity, but so far no tissue-protective effects have been reported. In contrast, blockade of IL-1 in rheumatoid arthritis patients, by an IL-1 receptor antagonist, was only moderately effective in suppressing inflammatory symptoms but appeared to reduce the rate of progression of joint destruction. We therefore used an established collagen II murine arthritis model (collagen-induced arthritis(CIA)) to study effects on joint structures of neutralization of either TNF-α or IL-1. Both soluble TNF binding protein and anti-IL-1 treatment ameliorated disease activity when applied shortly after onset of CIA. Serum analysis revealed that early anti-TNF-α treatment of CIA did not decrease the process in the cartilage, as indicated by the elevated COMP levels. In contrast, anti-IL-1 treatment of established CIA normalized COMP levels, apparently alleviating the process in the tissue. Histology of knee and ankle joints corroborated the finding and showed that cartilage and joint destruction was significantly decreased after anti-IL-1 treatment but was hardly affected by anti-TNF-α treatment. Radiographic analysis of knee and ankle joints revealed that bone erosions were prevented by anti-IL-1 treatment, whereas the anti-TNF-α-treated animals exhibited changes comparable to the controls. In line with these findings, metalloproteinase activity, visualized by VDIPEN production, was almost absent throughout the cartilage layers in anti-IL-1-treated animals, whereas massive VDIPEN appearance was found in control and sTNFbp-treated mice. These results indicate that blocking of IL-1 is a cartilage- and bone-protective therapy in destructive arthritis, whereas the TNF-α antagonist has little effect on tissue destruction. The Journal of Immunology, 1999, 163: 5049–5055.

Inflammation or clinical disease activity appears not necessarily linked to progression of joint destruction (7). Studies of the latter component of the disease is difficult to document in patients, short of using extremely long trials. Therefore we turned to animal models to allow documentation of all parameters, including the process in the joint structures. Murine collagen-induced arthritis (CIA) is a widely used experimental model of arthritis and has histopathological features in common with RA. It has been shown that neutralization of TNF-α ameliorates disease activity when administered before or shortly after onset of the disease (8–10). However, anti-TNF-α treatment did not decrease disease activity when given during established CIA. Blocking of IL-1, either after onset or during established CIA, effectively suppresses the arthritic process, recently demonstrated in several studies (10, 11).

We now investigated whether neutralization of the monokines TNF-α or IL-1 during CIA influences the development of cartilage and bone destruction. Treatment with either soluble TNF binding protein or anti-IL-1α+β was started shortly after onset of disease, and clinical as well as effects on the tissues were monitored. Serum cartilage oligomeric matrix protein levels were analyzed as a marker of cartilage pathological turnover, and radiography was used to determine bone destruction. Expression of the aggrecan neoeptope VDIPEN in the cartilage layers, as marker of metalloprotease activity, potentially a component in the destructive process, was demonstrated by immunohistochemistry. Furthermore, histology of knee and ankle joints was performed to investigate the effect of anti-TNF-α or anti-IL-1α+β treatment on the joint tissues.

The findings in this study showed that both anti-TNF-α and anti-IL-1α+β treatment ameliorates the inflammatory component of the disease but only neutralization of IL-1α+β prevents cartilage and bone destruction in CIA.
Materials and Methods

Animals

Male DBA-1 mice were obtained from Bomholtgard, Rye, Denmark. Mice were kept in filter top cages and were fed a standard diet and tap water ad libitum. Mice were used at 10 to 12 wk of age.

Collagen arthritis induction and anti-cytokine treatment

Mice were immunized with 100 μg bovine type II collagen in CFA enriched with Mycobacterium tuberculosis H37Ra (4 mg/ml) at the base of the tail. Bovine collagen was isolated as described elsewhere (12). The mice were boostered i.p. with 100 μg collagen dissolved in saline. After disease onset at day 28, mice were selected and divided into separate groups of at least 10 mice. The mean arthritis score of the control and anti-cytokine groups was comparable at the start of treatment. To neutralize TNF-α, mice were injected i.p. every other day with 3 mg/kg dimerically linked PE-Glylated soluble p55 TNFRI receptor (Amgen, Boulder, CO). This so-called TNFbp showed efficacy in murine streptococcal cell wall arthritis (13). The 50% effective dose (ED_{50}) of TNFbp to block the cytotoxic effect of murine TNF-α (mTNF-α) in the L929 bioassay was 5 × 10^{-9} M. To eliminate IL-1α+β, mice received one single injection of purified (IgG fraction) rabbit anti-murine IL-1α and anti-IL-1β (1 mg of each Ig). This dose revealed to be sufficient to suppress several murine arthritis models such as Ag-induced arthritis, immune complex-induced arthritis, and CIA (10, 14–16). One microgram of these anti-IL-1 Abs neutralized 50–100 pg IL-1 in the NOB-1 bioassay. As control, we used either BSA (3 mg/kg) or normal rabbit IgG (2 mg i.p.).

Assessment of CIA

Mice were carefully examined three times a week for the visual appearance of arthritis in peripheral joints, and scores for disease activity were given as previously described (10, 14). The clinical severity of arthritis (arthritis score, Fig. 1) was graded on a scale of 0–2 for each paw, according to changes in redness and swelling. At later time points, ankylosis was included in the macroscopic scoring.

IL-6 bioassay

IL-6 activity was determined by a proliferative assay using B9 cells. Briefly, 5 × 10^3 B9 cells in 200 μl 5% FCS-RPMI 1640 medium per well were plated in a round-bottom microtiter plate and incubated for 3 days using human recombinant IL-6 (R&D Systems, Minneapolis, MN) as standards. At the end of the incubation, 0.5 μCi of [3H]thymidine (NEN-DuPont, Boston, MA) was added per well. Three hours later, cells were harvested, and thyroidine incorporation was determined. Detection limit for the IL-6 bioassay was 1 pg/ml.

Cartilage oligomeric matrix protein measurements

At the end of the experiments, serum samples were taken and murine cartilage oligomeric matrix protein (COMP) levels were determined by ELISA using similar conditions as described for the assay for human COMP (17). The assay was modified by using rat COMP for coating the microtiter plate and for the standard curve included in each plate as well as by using a polyclonal antiserum raised against rat COMP (18). A high cross-reactivity to murine COMP was shown both by parallel dilution curves of murine sera to the standard curve as well as by experiments where a dilution of murine serum was added to the standard curve.

Radiology and histology

Knee and ankle joints were removed at the end of the experiments and were fixed and used for radiographic analysis as a marker for bone destruction. Radiographs were carefully examined by using a stereo microscope. Joint destruction was scored on a scale from 0–5, where 0 denotes no damage; 1, minor bone destruction observed in one enlightened spot; 2, moderate changes, 2–4 spots in one area; 3, marked changes, 2–4 spots in more areas; 4, severe erosions affectting the joint; and 5, complete destruction of the joint. Radiographs were scored by two observers without knowledge of the experimental group. For histology, joints were decalcified, dehydrated, and embedded in paraffin (10). Standard sections of 7 μm were made and stained with either hematoxylin and eosin or safranin O. Serial sections were scored by two observers on decoded slides. Inflammation was graded on a scale from 0 (no inflammation) to 3 (severe inflamed joint) as influx of inflammatory cells in synovium and joint cavity. Cartilage destruction and matrix proteoglycan depletion were scored on a scale from 0–3 ranging from no abnormalities to completely destroyed or destained (with safranin O) cartilage. Bone erosions were graded on a scale 0–3, ranging from normal bone appearance to fully eroded cortical bone structure in patella and femur condyle (10, 14).

Immunohistochemical VDIPEN staining

For immunostaining, sections of knee joints were deparaffinized, rehydrated, and digested with proteinase-free chondroitinase ABC to remove the side chains of the proteoglycans. Subsequently, sections were treated with 1% hydrogen peroxide, 1.5% normal goat serum, and affinity-purified rabbit anti-VDIPEN IgG (kindly provided by Dr. I. I. Singer and Dr. E. K. Bayne, Merck, Rahway, NJ). This Ab has been characterized before (19, 20). Thereafter, sections were incubated with biotinylated goat anti-rabbit, and avidin-streptavidin-peroxidase (Elite kit, Vector Labs, Burlingham, CA) staining was performed. Counterstaining was done with orange G.

Statistical methods

The significance of difference between group means was determined by a Mann-Whitney U test in the program SigmaStat (SPSS Software Products, Chicago, IL.).

Results

Amelioration of clinical disease activity by both sTNFbp and anti-IL-1 treatment

Anti-TNF-α treatment by injection of sTNFbp ameliorated clinical expression of CIA when started shortly after disease onset (Fig. 1A). Significant reduction of joint swelling and redness was noted after 4 days of treatment. Blockade of IL-1 during established CIA resulted in marked suppression of clinical disease activity, as can be seen in Fig. 1B. Actually, one single injection of anti-IL-1α+β...
Abs was sufficient to reduce clinical signs. The antiinflammatory effect of both sTNFbp and anti-IL-1β was further demonstrated by the reduced serum IL-6 levels determined at the end of treatment (Fig. 2). IL-6 can be viewed as an acute phase protein as described previously (21). The strong reduction of serum IL-6 indicated that TNF-α was sufficiently neutralized by sTNFbp treatment. COMP, a circulating marker of cartilage turnover is decreased by anti-IL-1 treatment

To obtain further insight into the protection against cartilage destruction, we determined serum COMP levels in the various groups. COMP is released from cartilage as a result of increased turnover in human and experimental arthritis (17, 18, 22). Fig. 3A shows a strong correlation in disease with no intervention between clinical arthritis score and serum COMP levels ($r = 0.94$). This is in line with previous findings in collagen-induced and pristane-induced arthritis in rats (18, 23). Although treatment with sTNFbp suppressed clinical disease activity of the CIA, no reduction was found in serum COMP levels, indicating little effect on the process in the cartilage (Fig. 3B). In contrast, strong reduction of serum COMP levels was seen in the anti-IL-1α+β-treated animals (6.5 ± 0.9 μg/ml vs 3.4 ± 0.5 μg/ml).

Prevention of bone erosions by anti-IL-1 but not by sTNFbp treatment

Radiological analysis, as an indicator of bone erosions of knee and ankle joints, revealed that elimination of TNF-α in established CIA did not retard the radiological progression of bone destruction (Fig. 4). The lack of an effect on bone destruction was further illustrated in Fig. 4, which shows erosive processes in knee joints on femur and tibia. In contrast, anti-IL-1α+β treatment abolished bone erosions, as demonstrated in Fig. 4. Fig. 5 further emphasizes the protective effect of the anti IL-1 treatment.

Effect of neutralization of TNF-α or IL-1 on joint pathology as demonstrated by histological examination

To confirm that IL-1 has a key role in cartilage and bone destruction in joint disease, we graded pathology on sections of whole knee joints. Table I shows that sTNFbp treatment reduced the inflammatory process, determined as the number of cells in the synovium and joint cavity, but had only marginal effect on cartilage damage, matrix proteoglycan depletion, and bone erosions. This is further illustrated in Fig. 6. Almost complete prevention of

FIGURE 2. Serum IL-6 levels after blockade of either TNF-α or IL-1. IL-6 levels were determined at day 36 after treatment with either sTNFbp or anti-IL-1 by using B9-cell bioassay. For treatment protocol see Fig. 1. Data are expressed as mean ± SD of at least seven mice per group. *, $p < 0.01$, Mann-Whitney $U$ test, compared with arthritic vehicle-treated animals.

FIGURE 3. IL-1 neutralization reduced serum COMP levels. Serum COMP were determined in sera of arthritic mice expressing different disease activity at day 36 after immunization (A). Strong correlation was found between disease activity and circulating COMP levels, $r = 0.94$. B. Serum COMP levels, determined at day 36 after treatment with either sTNFbp or anti-IL-1α+β. For details see Fig. 1 and Materials and Methods. Dotted line indicates serum COMP level in normal DBA-1 mice (4.2 ± 0.6). *, $p < 0.001$, Mann-Whitney $U$ test, compared with control.

FIGURE 4. Effect of either anti-TNF-α or anti-IL-1α+β treatment on joint destruction. Joints were x-rayed on day 36 after induction of CIA. Destruction was graded from x-ray photography by scoring bone erosions on a scale from 0 (no alterations) to 5 (completely destroyed joints). For treatment protocol see Materials and Methods. Data represent the mean ± SD x-ray score of at least 20 joints. *, $p < 0.001$, Mann Whitney $U$ test compared with control.
cartilage and bone damage was achieved by anti IL-1α+β treatment during established CIA (Table I, Fig. 6).

Blocking of IL-1 prevents VDIPEN neoepitope expression

VDIPEN neoepitope is a marker of metalloproteinase (MMP)-mediated cleavage of aggrecan, the major proteoglycan of articular cartilage. Previous studies have revealed that VDIPEN neoepitope was more abundant at sites and stages of advanced damage (19). To further demonstrate the protective effects on cartilage by anti-IL-1α+β treatment, sections were stained for this neoepitope. As a typical example, VDIPEN was highly expressed throughout the cartilage layers of the patella and femur in sections of vehicle-treated animals (Fig. 7A). Elimination of TNF-α for 8 days, initiated shortly after onset of CIA, did not reduce VDIPEN neoepitope expression in cartilage, as can be seen in Fig. 7B. In contrast, VDIPEN expression was almost absent in cartilage of anti-IL-1α+β-treated animals (Fig. 7C).

Discussion

The onset of clinical symptoms and inflammation in collagen type II arthritis is TNF-α dependent, which is in line with a role of this cytokine also in human RA (24). Studies with neutralizing anti-TNF-α Abs or soluble TNF receptors have revealed a major suppressive effect of the clinical disease activity, when treatment was started directly after onset of CIA (8, 9). Recently, we showed that, when arthritis is fully expressed, subsequent blocking of TNF-α appeared only marginally effective, implying that TNF-α is crucial in onset but less important in propagation of arthritis (10). In contrast, neutralization of IL-1α+β in early and established stages of CIA markedly suppressed disease activity (10, 14). It has been shown that IL-1β is the pivotal cytokine in collagen type II arthritis regarding disease expression by using anti-IL-1β Abs in DBA-1 mice, by studies of CIA in IL-1β-deficient mice, and by administration of IL-1β-converting enzyme inhibitors (10, 14, 25, 26). Furthermore, it was showed that local inflammation was impaired in IL-1β-converting enzyme-deficient mice (27). In the present study, we investigated whether blocking of TNF-α or IL-1 during established CIA would, in addition to suppressing inflammatory disease activity, prevent tissue destruction. As demonstrated previously, anti-TNF-α treatment ameliorated CIA when started after onset of disease. Despite the fact that suppression of clinical appearance of CIA and reduced serum IL-6 levels were found by early anti-TNF treatment, serum COMP levels, cartilage damage, and bone destruction were not affected, showing that joint destruction is progressing. COMP is a major component of articular cartilage, and serum levels are considered as a marker of generalized cartilage turnover (17, 22). In joint disease, the contribution from other tissues, e.g. synovia, which has been shown to be capable of COMP production (28, 29), appears insignificant (T. Saxne and D. Heinegård, unpublished observations). Thus, we have found that serum COMP levels were increased after the occurrence of cartilage destruction in CIA in both rats and mice, while, in early stages

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Infiltration of Cells</th>
<th>Cartilage Damage</th>
<th>Proteoglycan Depletion</th>
<th>Bone Erosions</th>
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<td>Control (BSA)</td>
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<td>sTNFbp</td>
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<tr>
<td>Control (Ig)</td>
<td>1.8 ± 0.6</td>
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<td>2.1 ± 0.5</td>
<td>2.5 ± 0.9</td>
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<tr>
<td>Anti-IL-1α + β</td>
<td>0.4 ± 0.3*</td>
<td>0.4 ± 0.3*</td>
<td>0.5 ± 0.4*</td>
<td>0.6 ± 0.3*</td>
</tr>
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</table>

*Joint pathology was examined after treatment either with 3 mg/kg sTNFbp every other day or with one single injection of rabbit anti-IL-1α + β (1 mg each). Histology was performed on whole knee joints as described in Materials and Methods. Scoring was performed by two independent observers on decoded slides. *p < 0.001, Mann-Whitney U test, compared with controls.
of CIA with marked inflammation, no elevation of serum COMP was found (T. Saxne and L. A. B. Joosten, unpublished observations; E. Larsson and T. Saxne, unpublished observations). Corroborating the strong suppression of CIA and a cartilage protective effect by anti-IL-1α+β treatment, no elevated serum COMP levels were found in anti-IL-1-treated animals. This was further

FIGURE 6. Histopathology at day 36 of knee joints after treatment with either sTNFbp or anti-IL-1α+β. A. Severe inflammation and cartilage destruction (arrows) in control, BSA-treated animal. B. Complete loss of matrix proteoglycans, indicated by destained cartilage layers, in control group. C and D. Knee joint of an animal treated with sTNFbp. Although reduced infiltrate, no ameliorated cartilage pathology when compared with control. E and F. Almost homogeneous safranin O staining indicated a cartilage-protective therapy by anti-IL-1 treatment. Furthermore, markedly reduced inflammation by the latter treatment. No difference was seen between the two control groups. A, C, and E. Hematoxilin and eosin staining. B, D, and F. Safranin O staining. P = patella, F = femur, C = cartilage, JS = joint space. Original magnification ×100.
supported by VDIPEN neoepitope appearance in the cartilage layers. Marked presence of this neoepitope was found throughout the cartilage in control and sTNFbp-treated animals, whereas animals treated with anti-IL-1α+β had almost no VDIPEN neoepitope. This neoepitope is formed by proteolytic cleavage of aggrecan by metalloproteinases. The fragment remains attached to hyaluronan in the cartilage, as an indicator of the proteolytic activity (30). In the model of Ag-induced arthritis, we noted that VDIPEN expression depended primarily on effects of IL-1, and the location correlated with severe cartilage damage (19). More recently, it was demonstrated that VDIPEN expression reflects stromelysin (MMP-3) activity, since expression was absent in stromelysin-deficient mice (31).

Histological analysis of knee and ankle joints revealed that elimination of IL-1α+β, starting after onset of disease, reduced joint inflammation, cartilage damage, loss of matrix proteoglycan and bone erosions. The primary effect of treatment by sTNFbp injections was a decreased influx of inflammatory cells. Previous studies with anti-TNF-α Abs or sTNF receptor protein in CIA reported significant reduction of clinical disease activity, but hardly any measurable effect on cartilage or bone destruction (8, 9, 10). Although anti-TNF-α treatment was started directly after onset of disease, 75% of the animals developed moderate or severe cartilage erosions (8).

IL-1 appears to be an efficient mediator, since, in the zymosan-induced experimental arthritis, it is clearly demonstrated that IL-1 is responsible for the inhibition of chondrocyte proteoglycan synthesis. This suppressive effect appeared mediated by NO, since NOS2 gene knockout mice, while having inflammation, showed no inhibition of chondrocyte metabolism (32). Furthermore, it has been demonstrated that blocking of IL-1 activity prevented cartilage proteoglycan depletion in murine Ag-induced arthritis, indicating protection against cartilage damage (15). Interestingly, in this latter experimental arthritis, anti-IL-1 treatment did not ablate joint inflammation.

The first clinical trials with IL-1Ra in human RA demonstrated that IL-1Ra has a beneficial effect on the rate of progression of joint erosion as well as suppressing inflammatory disease activity (5, 6). Anti-TNF-α treatment on the other hand seems to be somewhat more effective in suppressing clinical disease activity, i.e., primarily inflammation. However, there are as yet no published data on effects of anti-TNF-α treatment on the progression of joint erosions (3, 4). We have preliminary data showing that, in RA patients treated with human anti-TNF-α (D2E7), serum COMP levels were not reduced, although impressive reduction of disease activity was seen. Furthermore, IL-1β expression in synovial biopsies was not changed by anti-TNF treatment. This argues against TNF-α-dependent IL-1 production in RA synovium (P. Barrera, L. A. B. Joosten, A. A. den Broeder, L. B. A. van de Putte, P. L. C. M. van Riel, and W. B. van den Berg, manuscript in preparation).

The present study indicates that blocking of IL-1 during arthritis represents therapy that protects the cartilage and bone structures. This is demonstrated by reduced serum COMP levels, reduced appearance of the VDIPEN neoepitope in cartilage, and joint histopathology with abolished erosions of the articular cartilage, as well as absence of radiographically detectable bone erosions. The effects of IL-1 inhibition, at the same time as TNF-α inhibition has little effect on tissue destruction, may appear puzzling. However, it is known that chondrocytes and osteoblasts may produce IL-1 (33). It is thus possible that the initial events include setting up a cycle with self-activated destruction in cartilage and bone. In view of these findings, although TNF-α inhibitors may relieve the actual clinical picture, the long-term outcome with regard to joint destruction is likely to have the same bad prognosis. Therefore, to suppress the inflammation and offer protection...
References


