Osteoclasts are essential for TNF-α–mediated joint destruction

Kurt Redlich,1 Silvia Hayer,2 Romeo Ricci,3 Jean-Pierre David,3 Makiyeh Tohidast-Akrad,4 George Kollias,5 Günter Steiner,1 Josef S. Smolen,1 Erwin F. Wagner,3 and Georg Schett1

1Department of Internal Medicine III, Division of Rheumatology, University of Vienna, Vienna, Austria
2Institute of Medical Biochemistry, Vienna Biocenter, Vienna, Austria
3Research Institute of Molecular Pathology, Vienna, Austria
4Ludwig Boltzmann Institute for Rheumatology, Vienna, Austria
5Institute of Immunology, Biomedical Science Research Center Alexander Fleming, Vari, Greece

The detailed cellular and molecular mechanisms leading to joint destruction in rheumatoid arthritis, a disease driven by proinflammatory cytokines, are still unknown. To address the question of whether osteoclasts play a pivotal role in this process, transgenic mice that express human TNF (hTNFtg) and that develop a severe and destructive arthritis were crossed with osteopetrotic, c-fos–/– completely lacking osteoclasts. The resulting mutant mice (c-fos–/– hTNFtg) developed a TNF-dependent arthritis in the absence of osteoclasts. All clinical features of arthritis, such as paw swelling and reduction of grip strength, progressed equally in both groups. Histological evaluation of joint sections revealed no difference in the extent of synovial inflammation, its cellular composition (except for the lack of osteoclasts), and the expression of matrix metalloproteinase-3 (MMP-3) and MMP-13. In addition, cartilage damage, proteoglycan loss, and MMP-3, -9, and -13 expression in chondrocytes were similar in hTNFtg and c-fos–/– hTNFtg mice. However, despite the presence of severe inflammatory changes, c-fos–/– hTNFtg mice were fully protected against bone destruction. These data reveal that TNF-dependent bone erosion is mediated by osteoclasts and that the absence of osteoclasts alters TNF-mediated arthritis from a destructive to a nondestructive arthritis. Therefore, in addition to the use of anti-inflammatory therapies, osteoclast inhibition could be beneficial for the treatment of rheumatoid arthritis.


Introduction
Rheumatoid arthritis (RA) is the most severe chronic joint disease by virtue of persistent inflammation and destruction of cartilage and bone. The latter is a characteristic feature of RA usually not observed in other forms of inflammatory arthritis and constitutes a major cause of progressive disability and crippling of RA patients (1). The mechanisms leading to joint destruction still have not been fully elucidated. As a typical histopathological feature, a hyperplastic and hypercellular synovial membrane is built up in which lymphocytes, macrophage-like cells, and fibroblast-like cells accumulate. Experimental evidence suggests that erosive arthritis caused by grafting human rheumatoid synovial membrane into the knee joints of SCID mice is dependent on macrophages and fibroblast-like cells (2). Furthermore, synovial fibroblasts express cathepsins, matrix metalloproteinases (MMPs) (3–5), and a vacuolar-type ATPase that could contribute to bone matrix degradation (6). The molecular players in RA are still poorly understood. Proinflammatory cytokines, in particular TNF-α and IL-1, have proven to be major contributors to this disease (7). In fact, mice overexpressing the proinflammatory cytokine TNF develop a destructive arthritis (8). Moreover, blocking TNF improves signs and symptoms of RA and arrests progression of erosions in a large number of RA patients (9) by reducing several cytokines and other inflammation-associated molecules (10). Nevertheless, many patients do not respond to inhibition of TNF, and in many the erosive process continues despite significant effects of anti-inflammatory therapies (9).

Recently the role of osteoclasts in bone destruction of RA has attracted growing interest (11). Osteoclast precursors and mature osteoclasts are abundant at sites of arthritic bone erosions (12, 13). Several studies have shown that both receptor activator of NF-κB ligand (RANKL), an essential factor for osteoclast differentiation (14, 15), and its receptor RANK, are
expressed on cells of the RA synovial membrane (16). However, RANKL also regulates interactions between T cells and dendritic cells. Local T cell activation is associated with the expression of RANKL and subsequently with joint destruction in an animal model of arthritis (17). In fact, administration of osteoprotegerin, a naturally occurring inhibitor of RANKL (14), leads to protection from bone destruction in experimental arthritis (17, 18). However, as RANKL and RANK are not involved only in osteoclast differentiation but also in other cell-cell interactions, including those of T cells and antigen-presenting cells, it is not clear on which particular cells the protective effects of osteoprotegerin or lack of RANKL on arthritic destruction are mainly exerted (17, 19, 20).

c-fos, a component of the dimeric transcription factor AP-1, is a key regulator of bone cell proliferation and differentiation (21). Mice transgenic for c-fos develop osteosarcomas (21), whereas c-fos knockout (c-fos−/−) mice are osteopetrotic (22). These c-fos mutant mice completely lack functional osteoclasts, but have increased numbers of bone marrow macrophages, whereas other hematopoietic lineages, including T cells, develop normally.

In the present study we investigated whether bone erosions can occur in an osteoclast-free model of arthritis. We generated c-fos−/− hTNF-transgenic (c-fos−/− hTNFtg) mice and provide evidence that arthritic erosions cannot develop in the absence of osteoclasts.

**Methods**

**Animals.** The heterozygous Tg197 TNF transgenic mice (C57/BL6) have been described previously (8). These mice develop a chronic inflammatory and destructive polyarthritis within 4–6 weeks of birth. c-fos−/− mice (C57/BL6/129sv/cp) were generated as previously described (23). hTNFtg and c-fos−/− mice were intercrossed to obtain double mutant animals. F2 generations were used and all data were generated from littermates. A total number of 39 mice (wild type, n = 5; hTNFtg, n = 17; c-fos−/−, n = 5; and c-fos−/− hTNFtg, n = 12) were investigated. c-fos−/− mice lack tooth eruption, therefore they had to be fed with a wet diet. For breeding purposes, hTNFtg mice were treated with 0.2 mg of an anti-TNF monoclonal antibody (Centocor Inc., Leiden, The Netherlands) twice weekly in order to prevent arthritis. All animal procedures were approved by the local ethical committee.

**Clinical assessment.** Clinical evaluation was started 4 weeks after birth and was performed weekly. Arthritis was evaluated in a blinded manner as described previously (24). Briefly, joint swelling was examined using a clinical score graded from 0 to 3 (0, no swelling; 1, mild swelling of toes and ankle; 2, moderate swelling of toes and ankle; 3, severe swelling of toes and ankle). In addition, grip strength of each paw was analyzed on a wire 3 mm in diameter, using a score from 0 to –4 (0, normal grip strength; –1, mildly reduced grip strength; –2, moderately reduced grip strength; –3, severely reduced grip strength; –4, no grip strength at all). Animals were sacrificed by cervical dislocation, the blood was withdrawn by heart puncture, and the paws of all animals were collected for histology. The latest evaluation was performed 10 weeks after birth.

**ELISAs.** Analysis of serum levels of hTNF and murine soluble TNF receptor type 1 (mTNF-R1) was performed by ELISA. Tests were established according to the manufacturer’s recommendations (R&D Systems Inc., Minneapolis, Minnesota, USA). The lowest levels of detection were 0.18 pg/ml for the hTNF ELISA and 5 pg/ml for the TNF-R1 ELISA, respectively.

**Histological assessment.** Hind and front paws and the right knees were fixed in 4.0% formalin overnight and then decalcified in 14% EDTA with pH adjusted to 7.2 by addition of ammonium hydroxide (Sigma-Aldrich, St. Louis, Missouri, USA) at 4°C until the bones were pliable. Serial paraffin sections (2 μm) of all four paws and the right knee joint were stained with hematoxylin and eosin (H&E), or with toluidine blue for tartrate-resistant acid phosphatase (TRAP) activity, and analyzed by immunohistochemical methods. TRAP staining was performed as previously described (16). For immunohistochemistry, deparaffinized, ethanol-dehydrated tissue sections were boiled for 2 minutes in 10 mM sodium citrate buffer (pH 6.0) using a 700-W microwave oven, then allowed to cool to room temperature and rinsed in detergent solution (0.5% Tween in PBS) for 10 minutes. Tissue sections were blocked for 20 minutes in PBS containing 20% rabbit serum followed by incubation for 1 hour at room temperature with the following antibodies: goat polyclonal anti–calcitonin receptor antibody (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA) diluted 1:500 in PBS containing 1% BSA (Sigma-Aldrich); rat monoclonal anti-fibroblast antibody (Biogenes, Poole, United Kingdom) diluted 1:40; rat monoclonal anti-macrophage (F4/80) antibody (Serotec Inc., Raleigh, North Carolina, USA) diluted 1:80; rat monoclonal anti-c-fos antibody (Serotec Inc., Raleigh, North Carolina, USA) and mouse monoclonal anti-TRAP antibody (Serotec Inc., Raleigh, North Carolina, USA) diluted 1:40. The list of used antibodies is summarized in the Supporting Information.

**Immunohistochemistry**

Immunohistochemical analysis was performed as previously described (16). For immunohistochemistry, deparaffinized, ethanol-dehydrated tissue sections were boiled for 2 minutes in 10 mM sodium citrate buffer (pH 6.0) using a 700-W microwave oven, then allowed to cool to room temperature and rinsed in detergent solution (0.5% Tween in PBS) for 10 minutes. Tissue sections were blocked for 20 minutes in PBS containing 20% rabbit serum followed by incubation for 1 hour at room temperature with the following antibodies: goat polyclonal anti–calcitonin receptor antibody (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA) diluted 1:500 in PBS containing 1% BSA (Sigma-Aldrich); rat monoclonal anti-fibroblast antibody (Biogenes, Poole, United Kingdom) diluted 1:40; rat monoclonal anti-macrophage (F4/80) antibody (Serotec Inc., Raleigh, North Carolina, USA) diluted 1:80; rat monoclonal anti-c-fos antibody (Serotec Inc., Raleigh, North Carolina, USA) and mouse monoclonal anti-TRAP antibody (Serotec Inc., Raleigh, North Carolina, USA) diluted 1:40. The list of used antibodies is summarized in the Supporting Information.

**Table 1**

Cellular composition and MMP expression is not influenced by the absence of c-fos, despite the absence of osteoclasts and reduced MMP-9 expression.

<table>
<thead>
<tr>
<th></th>
<th>hTNFtg</th>
<th>c-fos−/− hTNFtg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibroblasts (anti-fibroblast)</td>
<td>42.5% ± 3.2%</td>
<td>42.6% ± 2.8%</td>
</tr>
<tr>
<td>Macrophages (F4/80)</td>
<td>49.0% ± 2.3%</td>
<td>46.2% ± 2.6%</td>
</tr>
<tr>
<td>T cells (anti-CD3)</td>
<td>3.3% ± 0.8%</td>
<td>2.6% ± 0.3%</td>
</tr>
<tr>
<td>Osteoclasts (TRAP+ MNCs)</td>
<td>2.2% ± 0.1%</td>
<td>0% ± 0%</td>
</tr>
<tr>
<td>MMP-3</td>
<td>32.8% ± 1.5%</td>
<td>32.3% ± 0.8%</td>
</tr>
<tr>
<td>MMP-9</td>
<td>35.2% ± 1.4%</td>
<td>20.0% ± 1.3%</td>
</tr>
<tr>
<td>MMP-13</td>
<td>35.0% ± 2.1%</td>
<td>38.0% ± 3.3%</td>
</tr>
</tbody>
</table>

The Journal of Clinical Investigation | November 2002 | Volume 110 | Number 10
anti-CD3 antibody (Novocastra Laboratories Inc., Newcastle, United Kingdom) diluted 1:100; mouse monoclonal anti-MMP-3; mouse monoclonal anti-MMP-9; and mouse monoclonal anti-MMP-13 antibody (all from Lab Vision Corp., Fremont, California, USA) diluted 1:100. After sections were rinsed, endogenous peroxidase was blocked with 0.3% hydrogen peroxide in Tris-buffered saline (10 mM Tris-HCl and 140 mM NaCl, pH 7.4) for 10 minutes. This was followed by 30 minutes of incubation with a biotinylated species-specific anti-IgG secondary antibody (Vector Laboratories Inc., Burlingame, California, USA). Then sections were incubated with the VECTASTAIN ABC reagent (Vector Laboratories Inc.) for another 30 minutes using 3,3-diaminobenzidine (Sigma-Aldrich) for the color reaction, resulting in brown staining of antigen-expressing cells.

For quantification of the areas of inflammation, H&E-stained sections (five per mouse) were evaluated. The sum of the areas of inflammation for each single mouse was calculated by evaluating all digital, carpal, and tarsal joints as well as the right knee joint. The same H&E-stained sections were analyzed as described above for quantification of erosions. The number of osteoclasts was counted as described above from TRAP-stained serial sections. Cartilage breakdown (i.e., proteoglycan loss and matrix dissolution) was determined from toluidine blue-stained serial sections by assessing cartilage area as well as a cartilage score according to the method of Joosten et al. (25), with modifications. Breakdown was scored on a scale from 0 to 3 (0, fully stained cartilage; 1, destained cartilage; 2, destained cartilage with synovial cell invasion; 3, complete loss of cartilage). Quantification of immunohistochemical sections was performed by counting both total cell numbers and the numbers of positively stained cells from at least three different sites of three individual sections per mouse. A total of 12 mice (n = 3 of each group) was included in this immunohistochemical analysis.

**Ex vivo osteoclastogenesis.** For RANKL-induced osteoclast differentiation, spleen cells were plated overnight and nonadherent cells were recovered and plated in 24-well plates (10⁶ cells per well) in the presence of 20 ng/ml M-CSF and 50 ng/ml RANKL (both from R&D Systems Inc.). Osteoclast differentiation was evaluated after 4 days of culture by TRAP staining using the Leukocyte Acid Phosphatase Kit from Sigma-Aldrich.

**Statistical analysis.** Data are shown as mean ± SEM. Group mean values were compared by two-tailed Student t test. For correlation analysis, the nonparametric Spearman test was used.

**Results**

c-fos⁻/⁻ hTNFtg mice show a significant reduction of body weight, are osteopetrotic, and display arthritis. In order to obtain an osteoclast-free model of TNF-dependent arthritis, the osteopetrotic c-fos mutant mice were crossed with hTNF-transgenic mice. The offspring of all four genotypes (wild-type, hTNFtg, c-fos⁻/⁻, and c-fos⁻/⁻ hTNFtg) were born at Mendelian frequency and were viable. Compared with the wild-type genotype, mice of all other genotypes showed a significant reduction in body weight, which was most prominently seen in c-fos⁻/⁻ hTNFtg mice (−43%) but was also found in c-fos⁻/⁻ (−23%) and hTNFtg mice (−16%). c-fos⁻/⁻ hTNFtg mice were toothless and displayed severe osteopetrosis as previously described for the c-fos⁻/⁻ mice (22).

**Clinical signs of arthritis are c-fos independent.** We next analyzed whether the absence of c-fos alters the clinical course of TNF-driven arthritis, indicated by increasing joint swelling and reduction of grip strength over time. Mice lacking the hTNF transgene (wild-type and c-fos−/− mice) showed no signs of joint swelling during the observation period (Figure 1a). In contrast, in hTNFtg mice, joint swelling started at week 4 (mean swelling score ± SEM, 0.1 ± 0.07) and increased weekly to a maximum at week 10 (1.6 ± 0.27).

**Figure 1**

There are no differences in the clinical course of arthritis between hTNFtg and c-fos⁻/⁻ hTNFtg mice. Clinical course of arthritis indicated by (a) joint swelling and (b) grip strength was assessed in wild-type mice (filled triangles), c-fos⁻/⁻ mice (open circles), hTNFtg mice (open diamonds), and c-fos⁻/⁻ hTNFtg mice (filled squares). Asterisks indicate a significant (P < 0.01) increase in joint swelling and decrease in grip strength in hTNFtg and c-fos⁻/⁻ hTNFtg mice compared with baseline and with wild-type and c-fos⁻/⁻ mice.
In the c-fos–/– hTNFtg group, joint swelling was also present as early as week 4 (0.5 ± 0.19) and increased significantly thereafter (1.8 ± 0.23). Joint swelling did not differ significantly between the two hTNFtg groups at any individual point in time, clearly indicating that c-fos is not required for the development of inflammatory arthritis.

Grip strength was assessed as a surrogate marker for joint function (Figure 1b). The grip strength of hTNFtg mice significantly decreased (week 4, –0.2 ± 0.09; week 10, –1.9 ± 0.23; \( P < 0.01 \)), showing that hTNFtg leads to progressive loss of joint function. As expected, mice lacking c-fos (c-fos–/– and c-fos–/– hTNFtg mice) had lower grip strengths on the basis of their skeletal abnormalities (23) compared with wild-type and hTNFtg mice. c-fos–/– hTNFtg animals showed reduced grip strength from the start of observation (week 4, –1.1 ± 0.15), with progressive deterioration continuing up to week 10 (–2.4 ± 0.22; \( P < 0.01 \)). There were no significant differences in grip strength between hTNFtg and c-fos–/– hTNFtg mice during later stages of arthritis. Thus, TNF-dependent development of clinical arthritis and functional impairment is not altered despite the absence of c-fos.

Histological examination revealed a complete lack of bone erosions in c-fos–/– hTNFtg mice. Based on the fact that c-fos–/– hTNFtg and hTNFtg mice developed similar clinical symptoms of arthritis, we further evaluated arthritic lesions by quantitative histological analysis to estimate the amount of inflammatory tissue and erosions.

Representative histologies of a digital joint are shown in Figure 2. hTNFtg mice developed not only intense inflammation, but also severe bone erosions (Figure 2a). In contrast, c-fos–/– hTNFtg mice, while showing a degree of inflammatory tissue similar to that in hTNFtg animals, had a highly conserved joint architecture, and no histological signs of bone erosions were seen (Figure 2b). Animals of both control groups, wild-type (Figure 2c) and c-fos–/– (Figure 2d), lacked any sign of joint inflammation or destruction. As previously described for c-fos–/– mice, c-fos–/– hTNFtg mice exhibited osteopetrosis with thickening of the trabecular bones and narrowing of the bone marrow cavity.

Quantification of inflammatory tissue in digital, carpal, and tarsal joints and the right knee joints revealed a similar degree of inflammation in hTNFtg mice (4.4 ± 0.5 mm²) and c-fos–/– hTNFtg mice (3.4 ± 0.4 mm²) (Figure 3a). However, quantitative evaluation of bone destruction in the same sections showed severe erosive changes in hTNFtg mice (1.2 ± 0.3 mm²), while bone erosions were completely absent in c-fos–/– hTNFtg mice (Figure 3b). Thus c-fos knockout mice are protected against TNF-induced bone erosion, but not inflammation.

Cellular composition and MMP expression in joints of c-fos–/– hTNFtg animals show no changes beyond the absence of osteoclasts and lower MMP-9 expression. To address whether the absence of c-fos alters inflammatory...
responses, the cellular composition and MMP expression of synovial inflammatory tissue was assessed in hTNFtg and c-fos−/−hTNFtg mice by immunohistochemistry (Table 1). Both cell numbers and distribution of distinct cell populations were similar among hTNFtg (fibroblasts, 42.5% ± 3.2%; macrophages, 49.0% ± 2.3%; T cells, 3.3% ± 0.8%) and c-fos−/−hTNFtg animals (fibroblasts, 42.6% ± 2.8%; macrophages, 46.2% ± 1.6%; T cells, 2.6% ± 0.3%). However, osteoclasts were found only in hTNFtg mice (2.2% ± 0.1%). Furthermore, the expression of MMP-3 (32.8% ± 1.5% vs. 32.3% ± 0.8%) and MMP-13 (35.0% ± 2.1% vs. 38.0% ± 3.3%) was unchanged. A large number of cells in c-fos−/−hTNFtg mice expressed MMP-9 (20.0% ± 1.3%), although this was lower than in hTNFtg animals (35.2% ± 1.4%; P < 0.05). This reduction must be seen in the light of the lack of osteoclasts, since MMP-9 is the key MMP of osteoclasts (3). Moreover, MMP-9 was similarly expressed by chondrocytes from hTNFtg and c-fos−/−hTNFtg mice (see below). Thus, with the exception of osteoclasts and MMP-9 expression, the cellular composition and MMP expression of synovial inflammatory tissue in c-fos−/−hTNFtg mice did not differ from that of their hTNFtg littermates with erosive arthritis.

The absence of c-fos has no influence on hTNF and mTNF-R1 levels. To assess whether the lack of bone erosions observed in c-fos−/−hTNFtg mice was due to a change in the level of expression of the hTNF transgene, all animal sera were screened for the amount of circulating hTNF. hTNF was detectable only in the serum of hTNFtg and c-fos−/−hTNFtg mice (Table 2). No significant difference in hTNF serum levels was found between the two groups, indicating that the absence of c-fos had no influence on the expression of hTNF. We also measured the serum levels of murine soluble mTNF-R1, since it mediates the activity of hTNF in hTNFtg mice, and found all four groups expressing TNF-R1 without significant differences (Table 2).

TNF and RANKL cannot rescue osteoclastogenesis in vitro in the absence of c-fos. To analyze whether the expression of hTNFtg or RANKL could rescue osteoclastogenesis in c-fos−/−deficient mice, spleen cells from c-fos−/−, hTNFtg, and c-fos−/−hTNFtg mice were cultured either with M-CSF or M-CSF and RANKL. When cultured with M-CSF and RANKL, no osteoclasts could be generated in vitro from either c-fos−/− spleen cells or c-fos−/−hTNFtg spleen cells (data not shown). In contrast, osteoclast formation was observed when hTNFtg spleen cells were cultured in the presence of M-CSF and RANKL, but not M-CSF alone. Thus, osteoclast differentiation is also impaired in c-fos−/−hTNFtg cells in vitro.

Complete absence of osteoclasts in c-fos−/−hTNFtg mice and strong correlation between the number of osteoclasts and the size of erosions in hTNFtg mice. The failure of spleen cells from c-fos−/−hTNFtg mice to differentiate into osteoclasts in vitro did not exclude the possibility that osteoclasts could be generated in these animals in vivo, particularly since TNF has been shown to be able to induce osteoclast differentiation even in the absence of RANKL in vitro (26). We therefore assessed the presence and number of osteoclasts in both hTNFtg and c-fos−/−hTNFtg mice. In hTNFtg mice, initial destruction of bone is usually observed at the cartilage-pannus junction, with subsequent progressive resorption of the subchondral bone (Figure 4a). TRAP staining of these areas revealed numerous mono- and multinucleated TRAP-positive cells. Whereas the mononuclear TRAP-positive cells were interspersed within the pannus, multinucleated TRAP-positive cells were almost exclusively found attached to the bone at resorption lacunae (Figure 4b). Analysis of serial sections revealed that TRAP-positive multinucleated cells (Figure 4c) also expressed the calcitonin receptor (Figure 4d).

Table 2
The absence of c-fos has no influence on levels of hTNF and TNF-R1

<table>
<thead>
<tr>
<th>Group</th>
<th>hTNF (ng/ml)</th>
<th>mTNF-R1 (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>ND</td>
<td>846 ± 38</td>
</tr>
<tr>
<td>c-fos−/−</td>
<td>ND</td>
<td>780 ± 160</td>
</tr>
<tr>
<td>hTNFtg</td>
<td>65 ± 8</td>
<td>1,106 ± 197</td>
</tr>
<tr>
<td>c-fos−/−hTNFtg</td>
<td>62 ± 10</td>
<td>913 ± 293</td>
</tr>
</tbody>
</table>

Serum levels of hTNF and mTNF-R1 were measured 10 weeks after birth at the end of the experiment in wild-type, c-fos−/−, hTNFtg, and c-fos−/−hTNFtg mice. Data are shown as mean ± SEM. ND, not detectable.
The areas of erosions given in mm\(^2\) were highly correlated to the numbers of osteoclasts per compartment (shown for wild-type, TRAP-positive multinucleated cells in all joints at erosion sites are rare, hTNF\(tg\)) and invasion of inflammatory tissue into the cartilage was down in arthritic joints (Table 3). Interestingly, direct absence of \(c-fos\) and proteoglycan content is reduced in cartilage of hTNF\(tg\) and cartilage destruction (cartilage score) revealed no significant difference in cartilage area between wild-type (mean ± SEM, 54.3 ± 9.6) but a complete lack of osteoclasts (mean ± SEM, 54.3 ± 9.6) but a complete lack of osteoclasts in \(c-fos^{-/-}\) mice (Figure 5a). In fact, there was a highly significant correlation between osteoclast numbers and the area size of erosions (\(r = 0.9964; P > 0.0001\), Figure 5b).

Proteoglycan content is reduced in cartilage of hTNF\(tg\) and \(c-fos^{-/-}\) mice. We also investigated whether the absence of \(c-fos\) had an influence on cartilage breakdown in arthritic joints (Table 3). Interestingly, direct invasion of inflammatory tissue into the cartilage was rare in hTNF\(tg\) and \(c-fos^{-/-}\) mice. However, in hTNF\(tg\) mice, while the cartilage maintained its architecture, the underlying bone underwent rapid resorption. Quantitative assessment of cartilage destruction revealed no significant difference in cartilage area between wild-type (mean ± SEM, 1.9 ± 0.3 mm\(^2\)) and \(c-fos^{-/-}\) mice (2.1 ± 0.4 mm\(^2\)). Compared with these controls, both TNF\(tg\) groups of mice showed a trend toward lower amounts of cartilage, although this did not reach significance (hTNF\(tg\), 1.5 ± 0.2 mm\(^2\); \(c-fos^{-/-}\) hTNF\(tg\), 1.7 ± 0.2 mm\(^2\)). However, qualitative assessment of cartilage breakdown including proteoglycan loss, synovial cell invasion, and cartilage destruction (cartilage score) revealed significant alterations of cartilage in both hTNF\(tg\) and \(c-fos^{-/-}\) hTNF\(tg\) mice compared with wild-type and \(c-fos^{-/-}\) controls. In fact, hTNF\(tg\) and \(c-fos^{-/-}\) hTNF\(tg\) mice did not significantly differ in their cartilage scores (Table 3). These findings are illustrated by toluidine blue staining of articular cartilage showing a marked reduction of proteoglycan content, predominantly at sites adjacent to inflammatory tissue in both hTNF\(tg\) (Figure 6a) and \(c-fos^{-/-}\) hTNF\(tg\) mice compared with controls (Figure 6, c and d). The capacity of TNF to induce MMP expression in chondrocytes was not altered by the deficiency of \(c-fos\), since MMP-3, MMP-9, and MMP-13 were similarly induced in hTNF\(tg\) and \(c-fos^{-/-}\) hTNF\(tg\) mice (Table 3). This suggests that independent of \(c-fos\), the inflammatory process led to significant induction of MMP expression and degradation of cartilage matrix.

**Figure 5**
Size of bone erosions is strongly correlated to number of osteoclasts. Numbers of osteoclasts assessed by histological quantification of TRAP-positive multinucleated cells in all joints at erosion sites are shown for wild-type, \(c-fos^{-/-}\), hTNF\(tg\), and \(c-fos^{-/-}\) hTNF\(tg\) mice (a). The areas of erosions given in mm\(^2\) were highly correlated to the numbers of osteoclasts per compartment (b).

Detailed quantitative analyses revealed a large number of osteoclasts at erosion sites in hTNF\(tg\) mice (mean ± SEM, 54.3 ± 9.6) but a complete lack of osteoclasts in \(c-fos^{-/-}\) hTNF\(tg\) mice (Figure 5a). In fact, there was a highly significant correlation between osteoclast numbers and the area size of erosions (\(r = 0.9964; P > 0.0001\), Figure 5b).

Proteoglycan content is reduced in cartilage of hTNF\(tg\) and \(c-fos^{-/-}\) hTNF\(tg\) mice. We also investigated whether the absence of \(c-fos\) had an influence on cartilage breakdown in arthritic joints (Table 3). Interestingly, direct invasion of inflammatory tissue into the cartilage was rarely observed in hTNF\(tg\) and \(c-fos^{-/-}\) hTNF\(tg\) mice. However, in hTNF\(tg\) mice, while the cartilage maintained its architecture, the underlying bone underwent rapid resorption. Quantitative assessment of cartilage destruction revealed no significant difference in cartilage area between wild-type (mean ± SEM, 1.9 ± 0.3 mm\(^2\)) and \(c-fos^{-/-}\) mice (2.1 ± 0.4 mm\(^2\)). Compared with these controls, both TNF\(tg\) groups of mice showed a trend toward lower amounts of cartilage, although this did not reach significance (hTNF\(tg\), 1.5 ± 0.2 mm\(^2\); \(c-fos^{-/-}\) hTNF\(tg\), 1.7 ± 0.2 mm\(^2\)). However, qualitative assessment of cartilage breakdown including proteoglycan loss, synovial cell invasion, and cartilage destruction (cartilage score) revealed significant alterations of cartilage in both hTNF\(tg\) and \(c-fos^{-/-}\) hTNF\(tg\) mice compared with wild-type and \(c-fos^{-/-}\) controls. In fact, hTNF\(tg\) and \(c-fos^{-/-}\) hTNF\(tg\) mice did not significantly differ in their cartilage scores (Table 3). These findings are illustrated by toluidine blue staining of articular cartilage showing a marked reduction of proteoglycan content, predominantly at sites adjacent to inflammatory tissue in both hTNF\(tg\) (Figure 6a) and \(c-fos^{-/-}\) hTNF\(tg\) (Figure 6b) mice compared with controls (Figure 6, c and d). The capacity of TNF to induce MMP expression in chondrocytes was not altered by the deficiency of \(c-fos\), since MMP-3, MMP-9, and MMP-13 were similarly induced in hTNF\(tg\) and \(c-fos^{-/-}\) hTNF\(tg\) mice (Table 3). This suggests that independent of \(c-fos\), the inflammatory process led to significant induction of MMP expression and degradation of cartilage matrix.

**Table 3**
Cartilage breakdown is dependent on TNF but not \(c-fos\)

<table>
<thead>
<tr>
<th>Group</th>
<th>WT (mean ± SEM)</th>
<th>(c-fos^{-/-}) (mean ± SEM)</th>
<th>hTNF(tg) (mean ± SEM)</th>
<th>(c-fos^{-/-}) hTNF(tg) (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cartilage area</td>
<td>2.1 ± 0.4</td>
<td>1.5 ± 0.2</td>
<td>1.7 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Cartilage score</td>
<td>0.1 ± 0.1</td>
<td>1.4 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>MMP-3</td>
<td>0</td>
<td>33.3 ± 5.2</td>
<td>33.0 ± 3.0</td>
<td></td>
</tr>
<tr>
<td>MMP-9</td>
<td>0</td>
<td>20.5 ± 1.5</td>
<td>22.0 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>MMP-13</td>
<td>0</td>
<td>36.3 ± 4.3</td>
<td>35.7 ± 1.8</td>
<td></td>
</tr>
</tbody>
</table>

\(\Delta\) Quantitative assessment of cartilage area (mean ± SEM in mm\(^2\)). Cartilage scores were determined using an arbitrary scoring ranging from 0 to 3 (0, fully stained cartilage; 1, destained cartilage; 2, destained cartilage with synovial cell invasion; 3, complete loss of cartilage). Analyses were performed with toluidine blue-stained sections. For cartilage staining with MMP-3, MMP-9, and MMP-13 in hTNF\(tg\) and \(c-fos^{-/-}\) hTNF\(tg\) mice, percentages of positively stained chondrocytes are given. All data are shown as mean ± SEM.
Several experimental animal models of arthritis indicate an important role for osteoclasts in local bone erosion. Treatment with osteoprotegerin prevents local bone erosion in adjuvant arthritis (17) by blocking the osteoclastogenic properties of activated RANKL-expressing T cells. Furthermore, osteoprotegerin significantly reduces local bone erosion in a TNF-driven model of arthritis (18), which is T cell– and B cell–independent, suggesting that RANKL expression by immunocompetent cells is not a paradigm for osteoclast-mediated local bone resorption. Further evidence comes from the study of osteoclast-free RANKL–/– mice, which are almost completely protected from local bone erosion but not from arthritis induced by serum transfer from K/BxN mice (37). This latter model is T cell– and B cell–independent, although immune-complex mediated. All these models thus suggest that intact RANKL/RANK interaction is of central importance for osteoclast-mediated local bone erosion.

However, RANKL/RANK signaling is not confined to osteoclast development but is also important in the interaction of T cells with dendritic cells (38), and the effect of a blockade of RANKL/RANK signaling on these dendritic cell functions and antigen presentation is unclear. In fact, most experimental models of RA are dependent on immunocompetent cells or their products. Furthermore, there is experimental evidence for RANKL/RANK-independent mechanisms of osteoclastogenesis; in particular, TNF and IL-1 may be involved in such situations (26).

The c-fos–/– hTNFtg arthritis model allows definitive conclusions on the role of osteoclasts in destructive arthritis for several reasons. Our model is distinct from autoimmune models of arthritis, since it does not depend on autoantibodies, immune complexes, or T cells, but rather on cytokine-driven inflammation (39). The knockout system used selectively inhibits osteoclast differentiation (40). If TNF had intrinsic osteoclastogenic properties mediated through signaling via TNF-R1 (41) and independent of the RANKL/RANK system (26), the overexpression of TNF in c-fos–/– hTNFtg mice would have allowed observation of such effects. In contrast, similar to c-fos–/– mice, c-fos–/– hTNFtg mice were protected from local bone erosion (27), and thus the infiltration of synovial inflammatory tissue into bone has long been considered a pathogenetic mechanism of bone erosion. Moreover, experimental evidence has suggested that cells, especially activated fibroblasts within the synovial membrane, may directly participate in the invasion (2) and resorption of bone in RA. On the other hand, the cell typically designed for bone resorption is the osteoclast, which harbors a number of specific tools for this special purpose (28, 29).

Therefore, there has been growing scientific interest in the hypothetical responsibility of osteoclasts for local bone resorption in arthritis (30). Indeed, the whole range of signals essential for osteoclastogenesis is found overexpressed in human RA. Aside from the well-known presence of TNF and IL-1 (31, 32), osteoclastic cytokines such as M-CSF and RANKL (in combination with its receptor RANK) are found in synovial inflammatory tissue (33). Furthermore, RA synovial tissue can be considered a suitable microenvironment for osteoclastogenesis, since synovial T cells and fibroblasts express RANKL in situ (16, 17), potentially serving as nursing cells (34), and a large number of cells of the monocyte/macrophage lineage, possibly serving as osteoclast progenitors, accumulate in the inflammatory lesions of RA (35). In fact, both osteoclast progenitors and mature osteoclasts have been described as being present at the site of bone erosions in human RA (12, 13, 36). Nevertheless, linking the mere presence of osteoclasts at the site of bone erosion to an essential role in inducing these lesions appeared far more difficult.
somewhat surprising. Although several signaling steps, such as MAPK/SAPK kinase activation (43), c-jun/c-fos overexpression (44), and increased AP-1 binding to DNA (45) have been described in human RA and animal models of RA, our data indicate that c-fos is not an essential part of inflammatory signaling of TNF-mediated arthritis.

Our findings clearly separate the mechanisms involved in joint inflammation and those of local bone destruction. The pathways leading to cartilage destruction, however, may be different, and much less is known about how to differentiate cartilage breakdown from bone erosion and/or inflammation. In the adjuvant arthritis model described by Kong et al. (17), osteoprotegerin treatment not only protected rats from bone damage but also from cartilage breakdown, suggesting that these two pathomechanisms are linked to each other. In contrast, in the serum transfer model described by Pettit et al. (37), significant proteoglycan loss and cartilage destruction was evident in the absence of local bone erosion. Thus mechanisms independent of RANKL-driven and osteoclast-mediated bone erosion, which induce cartilage breakdown, have to be postulated. Similarly, in the present model, lack of c-fos did not reduce inflammation-mediated changes of cartilage. Cartilage damage, as indicated by proteoglycan loss, cartilage destruction, and increased MMP-3, MMP-9, and MMP-13 expression in chondrocytes, was similar in hTNFtg and c-fos−/−hTNFtg mice. This clearly demonstrates that targeting of c-fos uncouples cartilage damage from bone erosion in this TNF-driven model of arthritis. Interestingly, the amount of cartilage destruction due to invasion of inflammatory tissue was relatively low compared with findings in the K/BxN serum transfer arthritis in RANKL−/−mice, in which adhesion to and invasion of the cartilage surface is far more pronounced (37).

In summary, we have shown that osteoclasts are the essential link between synovial inflammation and bone destruction. Despite severe synovial inflammation triggered by the overexpression of proinflammatory cytokines, articular bone remains well preserved in the absence of osteoclasts. Thus, inflammatory synovitis and joint destruction are two different processes, and inhibition of osteoclast differentiation and/or activation changes a destructive arthritis to a nondestructive form. Therefore these findings have important therapeutic implications: since the osteoclast renders chronic inflammation of the joint erosive, future therapies of destructive arthritis will have to take inhibition of osteoclasts into consideration.

Acknowledgments
This study was supported by the START price of the Austrian Ministry for Education, Science and Culture and the City of Vienna, and a grant from the Austrian National Bank (project 8715, G. Schett). The Research Institute of Molecular Pathology is supported by Boehinger Ingelheim.

osteoclast differentiation by a mechanism independent of the ODF/RANKL-RANK interaction.


