Imbalance of local bone metabolism in inflammatory arthritis and its reversal upon tumor necrosis factor blockade: direct analysis of bone turnover in murine arthritis

Jochen Zwerina, Birgit Tuerk, Kurt Redlich, Josef S Smolen and Georg Schett

Division of Rheumatology, Department of Internal Medicine III, Medical University of Vienna, Waehringer Guertel 18-20, A-1090 Vienna, Austria

Corresponding author: Georg Schett, georg.schett@meduniwien.ac.at

Received: 17 Aug 2005 Revisions requested: 26 Sep 2005 Revisions received: 18 Oct 2005 Accepted: 25 Nov 2005 Published: 30 Dec 2005

Arthritis Research & Therapy 2006, 8:R22 (doi:10.1186/ar1872)

This article is online at: http://arthritis-research.com/content/8/1/R22

© 2005 Zwerina et al.; licensee BioMed Central Ltd.

This is an open access article distributed under the terms of the Creative Commons Attribution License http://creativecommons.org/licenses/by/2.0, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Abstract

Chronic arthritis typically leads to loss of periarticular bone, which results from an imbalance between bone formation and bone resorption. Recent research has focused on the role of osteoclastogenesis and bone resorption in arthritis. Bone resorption cannot be observed isolated, however, since it is closely linked to bone formation and altered bone formation may also affect inflammatory bone loss. To simultaneously assess bone resorption and bone formation in inflammatory arthritis, we developed a histological technique that allows visualization of osteoblast function by in-situ hybridization for osteocalcin and osteoclast function by histochemistry for tartrate-resistant acid phosphatase. Paw sections from human tumor necrosis factor transgenic mice, which develop an erosive arthritis, were analyzed at three different skeletal sites: subchondral bone erosions, adjacent cortical bone channels, and endosteal regions distant from bone erosions. In subchondral bone erosions, osteoclasts were far more common than osteoblasts. In contrast, cortical bone channels underneath subchondral bone erosions showed an accumulation of osteoclasts but also of functional osteoblasts resembling a status of high bone turnover. In contrast, more distant skeletal sites showed only very low bone turnover with few scattered osteoclasts and osteoblasts. Within subchondral bone erosions, osteoclasts populated the subchondral as well as the inner wall, whereas osteoblasts were almost exclusively found along the cortical surface. Blockade of tumor necrosis factor reversed the negative balance of bone turnover, leading to a reduction of osteoclast numbers and enhanced osteoblast numbers, whereas the blockade of osteoclastogenesis by osteoprotegerin also abrogated the osteoblastic response. These data indicate that bone resorption dominates at skeletal sites close to synovial inflammatory tissue, whereas bone formation is induced at more distant sites attempting to counter-regulate bone resorption.

Introduction

Rheumatoid arthritis (RA) is one of the most typical examples of a chronic inflammatory process, which leads to profound changes of the skeleton [1]. In fact, RA and other forms of chronic arthritis are major precipitators of bone loss. Structural skeletal damage plays a major role in the outcome of RA patients since functional disability is a result of accumulating changes of the joint architecture [2]. Typically, juxta-articular bone is the skeletal site most exposed to the chronically inflamed RA synovial membrane, which directly invades bone and leads to formation of local erosions. These local bone erosions are characteristic for RA and are part of the diagnostic criteria of the disease [3].

The underlying mechanisms leading to the excessive bone loss in RA are not fully understood, although some key interactions between inflammation and bone, such as the receptor activator of NF-κB ligand (RANKL), have been unraveled during recent years [4-6]. Since bone loss is always the result of a negative net balance of bone formation and resorption, mediators expressed within the synovial tissue are thought to induce a shift from bone formation to bone resorption. For

bp = base pair; H & E = haematoxylin and eosin; hTNFtg = transgenic for human tumor necrosis factor; M-CSF = macrophage stimulating factor; NF = nuclear factor; OPG = osteoprotegerin; PBS = phosphate buffered saline; RA = rheumatoid arthritis; RANKL = receptor activator of NF-κB ligand; TNF = tumor necrosis factor; TRAP = tartrate-resistant acidic phosphatase.
instance, tumor necrosis factor (TNF) enhances osteoclast formation, and thus bone resorption, but it has also negative effects on bone formation since it interferes with differentiation and metabolic activity of osteoblasts [7-9].

The pathological role of altered bone turnover in destructive arthritis is strongly supported by the detection of osteoclasts at sites of local bone erosion [5]. Kinetic studies in animal models have also shown that osteoclast formation in the joint is a fast and dynamic process, leading to a rapid attack on juxta-articular bone, the prerequisite of early onset of structural damage [14]. Less is known about the role of osteoblasts in arthritic bone damage. These stromal-derived cells are the natural counterplayers of osteoclasts and might serve as a potential repair mechanism. In fact, osteoblasts are found within the arthritic bone erosions of animal models of arthritis as well as human RA [10]. In addition, stimulation of osteoblasts by parathyroid hormone has proven a repair process in experimental arthritis [15].

The relation of the metabolic activities of osteoclasts, evoking bone erosion, and of osteoblasts, mediating repair, is crucial for understanding the process of skeletal damage in arthritis. However, techniques that allow assessing the activity of both cell types simultaneously have not so far been employed. Here, using a new technique that facilitates simultaneous detection of metabolically active osteoclasts and osteoblasts in one histological section, and thus enables a direct investigation of bone turnover in arthritis, we have analyzed the role of osteoblasts relative to osteoclasts in experimental arthritis. As the experimental model for destructive arthritis we chose mice that are transgenic for human tumor necrosis factor (hTNFtg), since these mice develop a destructive arthritis closely mimicking human RA [16] and their disease is based on overexpression of TNF, which is centrally involved in joint inflammation and arthritic bone loss.

Materials and methods
Animals and treatments
The heterozygous hTNFtg mice (strain Tg197; genetic background, C57BL/6) have been described previously [16]. These mice develop a chronic inflammatory and destructive polyarthritis within 4–6 weeks after birth. In the present study, a total of 35 mice were examined in two independent experiments comprising 17 mice and 18 mice, respectively. The mice were divided into two groups in a 5:1:1 ratio and were treated according to the following protocol: group 1 received PBS (negative control); group 2 received a chimeric monoclonal anti-TNF antibody (infliximab, kindly provided by Centocor Pharmaceuticals, Leiden, The Netherlands) intraperitoneally at a dose of 10 mg/kg three times per week; and group 3 received osteoprotegerin (OPG) (an OPG-Fc fusion protein; Amgen, Thousand Oaks, CA, USA) intraperitoneally at a dose of 10 mg/kg three times a week. Therapy was started at the onset of symptoms (week 5) and lasted for 6 weeks. At the end of the study, mice were sacrificed by cervical dislocation, blood was drawn by heart puncture and the hind paws were obtained for histological evaluation. The local ethics committee approved all animal procedures.

Preparation of specimens for histological evaluation of bone erosions
Right hind paws were fixed in 4% paraformaldehyde overnight and were decalcified in 14% ethylenediamine tetraacetic acid (Sigma, St Louis, MO, USA) at 4°C (pH adjusted to 7.2 by addition of ammonium hydroxide; Sigma) until the bones were pliable. Serial paraffin sections (2 µm) were stained with H & E for evaluation of synovial inflammation and bone erosions.

Tartrate-resistant acidic phosphatase-osteocalcin double labeling
Double labeling of osteoclasts and osteoblasts was performed by combining tartrate-resistant acidic phosphatase (TRAP) labeling for detection of osteoclasts and osteocalcin in-situ hybridization for detection of osteoblasts. Sections were first stained with TRAP (leukocyte acid phosphatase kit; Sigma) as described previously [5]. The sections were subsequently prepared for in-situ hybridization by post-fixation of paw sections with 4% paraformaldehyde for 10 minutes at room temperature, rinsing with 0.1% Tween in PBS and digesting with proteinase K (1 µg/ml; Roche, Mannheim, Germany). After rinsing again with 0.1% Tween in PBS, another post-fixation step with 4% paraformaldehyde for 10 minutes at room temperature was carried out, followed by acetylation in acetic anhydride (2.5 µl/ml in triethanolamine buffer) for 15 minutes at room temperature and air drying for 30 minutes at 37°C.

The cDNA probe for the osteocalcin in-situ hybridization was kindly provided by Christine Hartmann (Research Institute of Molecular Pathology, Vienna, Austria). The osteocalcin probe consisted of a 600 bp insert cleaved with Nof and transcribed with T3 RNA polymerase to generate antisense probes using digoxigenin labeling (DIG RNA labeling kit; Roche). The digoxigenin-labeled osteocalcin probe was diluted 1:100 in hybridization solution (10 mM Tris, pH 7.5, 600 mM NaCl, 1 mM ethylenediamine tetraacetic acid, 0.25% SDS, 10% dextran sulfate, 1 × Denhardt’s solution, 200 µg/ml yeast tRNA, 50% formamide) and heated to 85°C for three minutes. The sections were then incubated with the probe, covered with coverslips and stored overnight in a humified chamber at 65°C. After removal of the coverslips, the slides were rinsed with 1 × SSC/50% formamide for 30 minutes at 65°C, followed by digestion of single-stranded RNA with RNase A (20 µg/ml in TNE buffer; Roche) for 10 minutes at 37°C and washing steps in TNE buffer (10 mM Tris, 500 mM NaCl, 1 mM
Detection of the probe was performed by incubating the slide with MABT buffer (100 mM maleic acid, 150 mM NaCl, 0.1% Tween 20, pH 7.5) for 5 minutes at room temperature before blocking with 20% heat-inactivated sheep serum in MABT buffer for 1 hour. The sections were then incubated with alkaline phosphatase-labeled anti-digoxigenin antibody (1:2000; Roche) in 2% heat-inactivated sheep serum/MABT buffer at 4°C in a humidified chamber. For the color reaction, nitroblue tetrazolium (0.25 µg/ml) and 5-bromo-4-chloro-3-indolyl phosphate (0.125 µg/ml) dissolved in NTMT buffer (100 mM NaCl, 100 mM Tris, pH 9.5, 50 mM MgCl₂, 0.1% Tween 20) was applied at room temperature overnight.

**Statistical analysis**

Data are presented as the mean ± standard error. Group mean values were compared by the two-tailed Student t test. The non-parametric Spearman test was used for correlation analysis.

**Results**

**Synovial inflammation affects juxta-articular bone at different compartments**

Based on the knowledge of arthritic bone loss in histological studies of animal models of arthritis and the radiographic appearance of arthritic bone erosions in human disease, we defined three different compartments of periarticular bone, where a direct in situ analysis of bone turnover would be of interest (Figure 1). The first is the cortical bone directly adjacent to articular cartilage at the cartilage-pannus junction (Figure 1b, black arrow), which is most directly affected by synovial inflammatory tissue leading to formation of subchondral bone erosions. Another compartment is the area of cortical bone channels, also termed Haversian channels (Figure 1b, red arrow), which is somewhat more distant, albeit still in close vicinity to the synovial inflammatory tissue. The final compartment is the endosteal surface of bone at sites distant from inflammatory tissue (Figure 1b, green arrows). We analyzed bone turnover in these compartments in hTNFtg mice, which develop a chronic destructive arthritis similar to RA. In this model, tarsal joints are among the first affected by arthritis, which is characterized by a hyperplastic synovial membrane invading the subchondral bone (Figure 1).

**Compartments with different patterns of bone turnover in inflammatory arthritis**

To assess bone turnover in the various juxta-articular bone compartments during arthritis, we developed a technique to simultaneously assess the metabolic activity of osteoclasts and osteoblasts in situ by detection of TRAP for osteoclast activity and of osteocalcin for osteoblast activity. Representative images of the different skeletal regions showing osteoclasts and osteoblasts simultaneously are depicted in Figure 2.

All hTNFtg mice showed accumulation of osteoclasts and osteoblasts in the inflamed joint region, whereas such cells were virtually absent in wild-type mice (data not shown). Within subchondral bone erosions, the number of osteoclasts significantly ($P < 0.01$) exceeded osteoblasts (mean ± standard error 13.6 ± 1.1 osteoclasts versus 2.5 ± 0.9 osteoblasts per mm bone surface; Figure 3a). Also, a fivefold larger bone surface was covered by osteoclasts (26.0 ± 2.1%) than by osteoblasts (5.4 ± 1.9%; Figure 3b). This indicates high bone turnover within subchondral bone with an excess in bone resorption. In cortical bone channels, however, we observed an equal distribution of osteoclasts and osteoblasts (9.5 ± 0.8 osteoclasts and 11.5 ± 1.1 osteoblasts per mm bone surface), both covering approximately 25% of the bone surface, indicating high but balanced bone turnover (Figure 3c,d). In contrast, the endosteal surface distant from bone erosions contained only few bone cells. Mean numbers of 1.2 (± 0.3) osteoclasts and 1.4 (± 0.4) osteoblasts per mm bone surface were detected, attributing to only 2.5% and 3.5% bone surface covered by osteoclasts and osteoblasts, respectively (Figure 3e,f). This indicates a low bone turnover at skeletal sites distant from bone erosions in inflammatory arthritis.

**Localization of osteoclasts and osteoblasts in local bone erosions of TNF transgenic mice**

Next, we determined the distribution of osteoclasts and osteoblasts in the primary skeletal lesion of arthritis: subchondral...
bone erosion. Three compartments were defined within these lesions: the surface adjacent to the articular cartilage (subchondral surface), the inner frontier of erosion (inner surface), and the surface adjacent to cortical bone (cortical surface).

Histomorphometrical analysis revealed that more than one-half of the osteoclasts (57%) were located at the subchondral bone surface, whereas fewer osteoclasts were found at the cortical bone surface (28%) and inner bone surface (15%), respectively (Figure 4a). The distribution of osteoblasts in subchondral bone erosions was inverse: most osteoblasts were found at the cortical bone surface (71%), fewer at the inner surface (29%), and no osteoblasts were seen at the subchondral surface of the erosions (Figure 4b). Similar results were also obtained when measuring the density of osteoclasts and osteoblasts at these different compartments: 53% of the subchondral bone surface was covered by osteoclasts, whereas none was covered by osteoblasts. At the inner bone surface, 21% and 5% bone was covered by osteoclasts and osteoblasts, respectively. In contrast, osteoblasts at the cortical bone surface covered a significantly greater amount of bone surface (21%) than osteoclasts (7%) (Figure 4c,d). Hence, osteoclasts are more frequent at the subchondral bone surface, whereas osteoblasts are most abundant at the cortical surface of bone erosions.

We next examined whether the size of subchondral bone erosions was linked to the amount of osteoclasts and osteoblasts within these lesions. A strong positive correlation ($r = 0.82; P < 0.001$) was found when correlating the number of osteoclasts with bone erosion size (Figure 4e). Interestingly, the number of osteoblasts at the site of bone erosion correlated...
inversely with the area of bone erosion \( r = -0.38, P < 0.05 \); Figure 4f).

**Reversed local bone turnover upon anti-TNF treatment in erosive arthritis**

We analyzed the effects of TNF blockade on local bone turnover. TNF blockade was initiated at the stage of early arthritis and was continued for six weeks. We then determined the extent of local bone damage and quantified the area of subchondral bone erosions. Whereas untreated TNF transgenic mice exhibited extensive bone erosions (mean ± standard error, 6330 ± 1060 µm²), anti-TNF treatment strongly reduced the area of bone erosions to 450 ± 170 µm² \( P < 0.001 \).

Moreover, the microenvironment in the residual subchondral bone erosions showed a dramatic change compared with untreated mice. As shown in Table 1, the number of osteoclasts was dramatically reduced to a mean of 1.9 ± 1.9 per bone perimeter (-86%), covering only 3 ± 3% of the bone surface (-88%) when compared with untreated animals, as...
shown in Figure 3). In contrast, the number of osteoblasts was strikingly increased to a mean of 47.9 ± 13.4 cells per bone perimeter (+1600%) resulting in 37% bone surface coverage by osteoblasts (+685%). A similar picture was observed when analyzing cortical bone channels. Osteoclasts decreased to a number of 8.9 ± 1.9 (-7%), covering 9% of the bone surface (-64%). At the same time, osteoblasts had expanded (26 ± 7.4 osteoblasts per mm bone perimeter [+226%]), reflecting 43% covered bone surface (+172%). At endosteal sites distant from bone erosions, the numbers of osteoclasts per bone perimeter remained unchanged (1.6 ± 0.7), covering 0.6% of the bone surface. The numbers of osteoblasts, however, increased to 11.2 ± 5.1 per bone perimeter, covering a mean of 4% of the endosteal bone surface.
TNF blockade therefore completely reverses increased bone resorption and leads to a dramatic increase in osteoblast numbers, indicating high bone turnover with a positive net balance in anti-TNF-treated arthritic mice.

Blockade of osteoclastogenesis abolishes osteoblast activity, indicating the osteoblastic response is dependent on bone erosion.

To determine whether specific inhibition of osteoclastogenesis without affecting synovial inflammation influences the anabolic response in bone of arthritic mice, we treated 6-week-old hTNFtg mice three times per week for 6 weeks with OPG. This treatment did not significantly inhibit joint inflammation (data not shown) but did strongly diminish local bone erosions by 90% (mean ± standard error, 6330 ± 1060 µm² for untreated mice versus 618 ± 860 µm² for OPG-treated mice) compared with TNF blockade. Analysis of osteoclasts and osteoblasts within the remaining bone erosions not only showed the anticipated dramatic reduction of osteoclasts in bone erosions (0.6 ± 0.4 osteoclasts per mm, -96%), but also a complete absence of osteoblasts (Table 1). A similar picture emerged when cortical bone channels were analyzed: almost no osteoclasts (0.4 ± 0.4 per mm bone surface) and almost no osteoblasts (0.2 ± 0.2 per mm bone surface) were detectable, attributing to less than 1% of a bone surface coverage by bone active cells. Similarly, no bone cells were seen at the endosteal bone surface distant from bone erosions.

Table 1

| Mice                  | OcPm/BPm (%) | ObPm/BPm (%) | NOc/BPm (cells/mm) | NOb/BPm (cells/mm) |
|-----------------------|--------------|--------------|--------------------|--------------------|
| Untreated hTNFtg      |              |              |                    |                    |
| Subchondral bone erosion 26 ± 2 | 5 ± 2        | 13.5 ± 1.1   | 2.5 ± 0.8          |
| Cortical bone channels 22 ± 1      | 25 ± 2       | 9.5 ± 0.7    | 11.4 ± 1.0         |
| Endosteal bone surface 1 ± 1        | 4 ± 1        | 1.2 ± 0.2    | 1.4 ± 0.3          |
| OPG-treated hTNFtg     |              |              |                    |                    |
| Subchondral bone erosion 1 ± 1        | 0            | 0.6 ± 0.4    | 0                  |
| Cortical bone channels 1 ± 1        | 1 ± 1        | 0.4 ± 0.4    | 0.2 ± 0.2          |
| Endosteal bone surface 0            | 0            | 0            | 0                  |
| Anti-TNF treated hTNFtg         |              |              |                    |                    |
| Subchondral bone erosion 3 ± 3        | 36 ± 5       | 4.1 ± 1.2    | 48.3 ± 11.4        |
| Cortical bone channels 9 ± 2        | 43 ± 10      | 8.7 ± 3.3    | 26.1 ± 7.2         |
| Endosteal bone surface 1 ± 1        | 4 ± 1        | 2.5 ± 1.2    | 10.9 ± 5.4         |

Results presented as the mean ± standard error of the mean. Paw sections of transgenic for human tumor necrosis factor (hTNFtg) mice treated for six weeks with a neutralizing antibody against TNF (anti-TNF) or osteoprotegerin (OPG) were stained for functional osteoclasts and osteoblasts. The fraction of bone surface covered by osteoclasts and osteoblasts (OcPm/BPm, ObPm/BPm) and the numbers of osteoclasts and osteoblasts per bone perimeter (NOc/BPm, NOb/BPm) were assessed and allocated to three skeletal compartments.

Selecting blockade of osteoclasts in arthritic mice is therefore accompanied by a strong reduction of osteoblasts, suggesting that accumulation of osteoblasts is a consequence of arthritic bone resorption and not of synovial inflammation per se.

Discussion

Skeletal turnover is a complex physiological process, which requires bone resorption mediated by osteoclasts as well as bone formation exerted by osteoblasts. Chronic inflammation can lead to sustained alterations of bone turnover, as typically seen in juxta-articular and periarticular damage of inflamed joints in RA. TNF is a proinflammatory cytokine of major importance in chronic inflammatory arthritis and also exerts profound effects on bone: TNF can induce osteoclast formation, whereas it has negative effects on bone formation. Its unfavorable skeletal effects on bone make TNF an ideal candidate for linking inflammation and bone loss. Indeed, the therapeutic blockade of TNF is highly effective in preventing and/or reducing skeletal damage in conditions of chronic arthritis [18].

In the current work, while developing a technique allowing direct visualization and quantitative assessment of metabolically active bone cells to simultaneously analyze functional osteoclasts and osteoblasts representing areas of bone loss and bone formation, respectively, we evaluated juxta-articular and periarticular bone turnover in the context of chronic arthritis. Using a model of TNF overexpression, these analyses revealed several interesting insights into arthritic bone loss, as illustrated in Figure 5.

First, bone turnover was massively increased at sites of joint inflammation. Arthritis in hTNFtg mice involves peripheral joints, such as tarsal, carpal and digital joints, largely reflecting...
the distribution of inflammation found in human disease. Small peripheral bones predominantly consist of cortical bone, which has a slow turnover compared with trabecular bone. In physiological conditions, therefore, only few osteoclasts and osteoblasts are found at these peripheral skeletal sites. In arthritis, however, accumulation of osteoclasts but also of osteoblasts is found at sites where these cells are normally absent or are only scarcely present [5]. On the basis of these data, the hypothesis that the inflammatory events in RA primarily lead to activation of osteoclasts and bone resorption by means of TNF and RANKL, but do not induce the osteoblast compartment and consequent bone formation, needs to be revisited.

While the negative effects of TNF on bone formation are well documented [8], synovial inflammation in fact appears to also provoke an osteoblastic response, which may be seen as an attempt at bone repair. In fact, the observed accumulation of osteoblasts may be considered as a skeletal response to TNF-mediated bone loss [19]. Osteoclasts and osteoblasts are in close vicinity to each other, suggesting a dynamic interplay between these cells, and consequently between bone resorption and formation at these skeletal sites. This hypothesis is supported by the observation of the metabolically active state of both cell types as observed by the expression of TRAP, a key enzyme for osteoclasts [20], as well as osteocalcin, a specific extracellular matrix molecule produced by osteoblasts [21]. Moreover, accumulation of both cell types was linked to synovial inflammatory tissue, since the number of active osteoclasts and osteoblasts decreased dramatically with growing distance from the inflamed joint, suggesting that mediators originating from inflammatory tissue influence activation of bone turnover by paracrine mechanisms.

A second insight is that the net effect on bone was highly dependent on the proximity to the inflamed synovial membrane. The area closest to inflammatory tissue is the subchondral bone of the junction zone, where the synovial membrane inserts into the cortical bone surface. This site faces rapid resorption, supported by the massive accumulation of osteoclasts, which by far outweighs the number of active osteoblasts in these lesions. The result is a negative net balance of bone formation compared with resorption and progressive local bone erosions. It also reflects the direct effects of a
chronic exposure of adjacent bone to proinflammatory cytokines from the synovial membrane and cells (osteoclasts) generated in the synovial membrane. In deeper skeletal layers, however, the osteoblastic response tends to outweigh bone resorption, as evident by similar numbers of osteoblasts and osteoclasts in cortical bone channels underneath erosions. These areas are characterized by high bone turnover with abundance of metabolically active osteoclasts and osteoblasts.

These differences between the compartments suggest that the inflammatory tissue directly drives osteoclastogenesis, which is a well-known process and involves the expression of TNF by various cells within inflammatory tissue and of RANKL by T cells and synovial fibroblasts [22-25]. On the other hand, these data show that the skeletal response to increased bone resorption emerges from deeper layers of the cortical bone and involves accumulation of osteoblasts synthesizing new bone matrix. A similar state is even found within subchondral bone erosions: although in these lesions only a minority of bone-active cells are osteoblasts, they are almost exclusively found in the deeper cortical areas surrounding the bone erosion but are absent in the superficial area adjacent to articular cartilage. This suggests that the catabolic signal for bone is strongly associated with inflammatory tissue, whereas the anabolic response comes from deeper layers of cortical bone.

Third, hypothesizing that the anabolic skeletal response is an indirect effect, which depends on bone resorption induced by synovial inflammatory tissue, we aimed to shut down osteoclasis specifically by interfering with RANKL-receptor activator of NF-κB interaction. Blockade of RANKL has proven to effectively prevent arthritic bone erosion without interfering with synovial inflammation [11,26-29]. Interestingly, the anabolic response was almost completely abolished when osteoclast generation was blocked by OPG. Previous studies assessing the systemic bone changes in hTNFtg mice have shown that OPG treatment not only blocks bone resorption, but also downregulates bone formation [19]. This is also in line with the effect of RANKL blockers in human clinical trials. The molecular background of the inhibitory effect of RANKL blockers in bone formation is poorly understood. It cannot be ruled out that osteoblastogenesis is dependent on RANKL; however, such an effect has only been described for glutathione S-transferase fusion RANKL (GST-RANKL), not for native RANKL [30].

More generally, however, these observations favor the concept that the generation of viable osteoclasts is necessary for optimal bone anabolic responses [31]. Such a hypothesis of coupling stress is also supported by recent studies, which suggest that acidification of the resorption compartment is an important stimulus for the influx of bone-forming cells into resorption sites [32]. An alternative explanation for osteoblastogenesis next to inflamed joints is that synovial inflammation is directly responsible for the anabolic response of cortical bone. However, several points of evidence contradict such a mechanism: proinflammatory mediators, such as TNF, are negative regulators and not positive regulators of osteoclasts; osteoclasts but not osteoblasts dominate the areas close to synovial inflammatory tissue; accumulation of osteoblasts is always linked to the presence of osteoclasts and is not seen as an isolated process upon emergence of arthritis; and selective blockade of bone resorption by RANKL blockade does not interfere with synovitis, but does block the anabolic response. This clearly indicates that the accumulation of metabolically active osteoclasts at sites of inflammatory bone erosions is not directly based on joint inflammation per se, but is rather a consequence of osteoclastogenesis and active bone resorption.

Fourth, from a therapeutic standpoint the reversibility of the pathophysiology of destructive arthritis is a key point of interest. Since in this model TNF is not only responsible for inflammatory arthritis but also for alteration bone turnover [19], removal of this essential trigger might also reverse dysregulation of bone turnover. Indeed, treatment with a TNF blocker completely reversed bone turnover by blocking osteoclasts and fostering osteoblasts. In subchondral sites of bone erosion, where osteoclasts dominated highly, TNF blockade completely switched negative bone turnover to an anabolic phenotype, leading to an excess amount of osteocalcin-synthesizing osteoblasts compared with osteoclasts. This was partially achieved by an effective inhibition of osteoclastogenesis, since TNF is not only a direct activator of osteoclasts but also induces expression of RANKL [9]. However, it was also based on an increase of osteoblasts, which illustrates the mentioned negative role of TNF on bone formation.

Whether these effects of TNF blockade on local bone metabolism are long-term effects is currently unknown, since the duration of treatment, although comparatively long for a model of inflammatory arthritis, is not designed to assess potential long-term effects. Another potential limitation is that no dose titration of skeletal effects of TNF and/or RANKL blockers has been accomplished, since blockade of TNF and RANKL was used as a proof-of-concept study on the role of TNF and osteoclasts in local skeletal remodeling in this experimental model.

**Conclusion**

This investigation extends current insights into bone loss in the context of inflammatory arthritis. Bone erosion is not a purely resorptive process, but rather harbors complex interactions of bone resorption and formation throughout the entire cortical bone. Induction of bone formation in deeper layers of cortical bone can be regarded as an attempt to auto-repair, which limits but does not reverse skeletal damage. This process depends on the potential of synovial inflammatory tissue to generate osteoclasts and to resorb bone, but is not a consequence of synovial inflammation per se. Most impor-
tantly, distorted bone metabolism is reversible when the initial trigger, primarily TNF, is neutralized — suggesting that TNF blockade might not only block bone resorption but also pave the path for effective repair of skeletal damage.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
JZ carried out breeding of the mice, performed histological analyses and drafted the manuscript. ET carried out histological analyses. KR and JSS participated in the design of the study. GS conceived the study, and participated in its design and coordination. All authors read and approved the final manuscript.

Acknowledgements
The authors thank Marco Koefe for excellent technical assistance and Dr Christine Hartmann (Research Institute of Molecular Pathology, Vienna, Austria) for providing the osteocalcin in-situ hybridization probe. They are grateful to Dr Giorgios Kollias (Alexander Fleming Biomedical Sciences Research Center, Vari, Greece) for providing breeding pairs of the hTNFtg mice. This study was supported by the START price of the Austrian Science Fund (GS).

References
1. Goldring SR: Bone and joint destruction in rheumatoid arthritis: what is really happening? J Rheumatol Suppl 2002, 65:44-48.
2. Drossaers-Bakker KW, de Buck M, van Zeben D, Zwinderman AH, Breedveld FC, Hazes JM: Long-term course and outcome of functional capacity in rheumatoid arthritis: the effect of disease activity and radiologic damage over time. Arthritis Rheum 1999, 42:1854-1860.
3. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, Healey LA, Kaplan SR, Ligthart SH, et al.: The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988, 31:315-324.
4. Shigeyama Y, Pap T, Kunzer P, Simmen BR, Gay RE, Gay S: Expression of osteoclast differentiation factor in rheumatoid arthritis. Arthritis Rheum 2000, 43:2523-2530.
5. Gravallese EM, Manning C, Tsay A, Naito A, Pan C, Amento E, Goldring SR: Synovial tissue in rheumatoid arthritis is a source of osteoclast differentiation factor. Arthritis Rheum 2000, 43:250-258.
6. Kong Y, Yoshida H, Sarosi I, Tan HL, Timms E, Capparelli C, Georghiou S, Kaslauskas R, Goldring SR, Choi Y, Benuist C, Gravallese EM: TRANCE/RANKL knockout mice are protected from bone erosion in a serum transfer model of arthritis. Am J Pathol 2001, 159:1689-1699.
7. Redlich K, Hayer S, Ricci R, David JP, Tohidast-Akrad M, Kollias G, Steiner G, Smolen JS, Wagner EF, Schett G: Osteoclasts are essential for TNF-alpha-mediated joint destruction. J Clin Invest 2002, 110:1419-1427.
8. Lubberts E, Oppers-Walgreen B, Petit AR, Van Den Besselaar L, Joosten LA, Goldring SR, Gravallese EM, Van Den Berg WB: Increase in expression of receptor activator of nuclear factor kappab at sites of bone erosion correlates with progression of inflammation in evolving collagen-induced arthritis. Arthritis Rheum 2002, 46:3035-3044.
9. Schett G, Stolina M, Bolon B, Middleton S, Adlam M, Brown H, Zhu L, Feige U, Zack DJ: Analysis of the kinetics of osteoclastogenesis in arthritic rats. Arthritis Rheum 2005, 52:3192-3201.
10. Redlich K, Gortz B, Hayer S, Zwirner J, Dooler N, Kostenuik P, Burgmair J, Kollas G, Schett G: Repair of local bone erosions and reversal of systemic bone loss upon therapy with anti-tumor necrosis factor in combination with osteoprotegerin or parathyroid hormone in tumor necrosis factor-mediated arthritis. Am J Pathol 2004, 164:543-555.
11. Schett G, Probert L, Georgopoulos S, Kaslauskas R, Goldring SR, Gravallese EM, Colias JZ, Kollas G: Transgenic mice expressing human tumour necrosis factor: a predictive genetic model of arthritis. EMBO J 1991, 10:4025-4031.
12. Gortz B, Hayer S, Redlich K, Zwirner J, Tohidast-Akrad M, Tuerk B, Hartmann C, Kollas G, Smolen JS, Schett G: Arthritis induces lympohocytic bone marrow inflammation and endostal bone formation. J Bone Miner Res 2004, 19:990-998.
13. Smolen JS, Han C, Bala M, Maini RN, Kalden JR, van der Heijde D, Breedveld FC, Furtst DE, Lipsky PE, ATTRACT study group: Evidence of radiographic benefit of treatment with infliximab plus methotrexate in rheumatoid arthritis patients who had no clinical improvement: a detailed subanalysis of data from the anti-tumor necrosis factor trial in rheumatoid arthritis with concomitant therapy study. Arthritis Rheum 2005, 52:1020-1030.
14. Schett G, Redlich K, Hayer S, Zwirner J, Tohidast-Akrad M, Lang C, Gortz B, Schulz A, Bergmeister H, Kollas G, et al.: Osteoprotegerin protects against generalized bone loss in tumor necrosis factor transgenic mice. Arthritis Rheum 2003, 48:2042-2051.
15. Hayman AR, Cox TM: Tartrate-resistant acid phosphatase knockout mice. J Bone Miner Res 2003, 18:1905-1907.
16. Brown JP, Delmas PD, Malaval L, Edouard C, Chapuy MC, Meunier PJ: Serum bone Gla-protein: a specific marker for bone formation in postmenopausal osteoporosis. Lancet 1984, 1:1091-1093.
17. MacNaul KL, Hutchinson NJ, Parsons JS, Bayne EK, Tocci MJ: Analysis of IL-1 and TNF-alpha gene expression in human rheumatoid synovocytes and normal monocytes by in situ hybridization. J Immunol 1990, 145:1451-1456.
18. Chu CC, Field M, Feldmann M, Maini RN: Localisation of tumour necrosis factor alpha in synovial tissues and at the cartilage-pannus junction in patients with rheumatoid arthritis. Arthritis Rheum 1991, 34:1125-1132.
19. Takayanagi H, Izuka H, Jujo T, Nakagawa T, Yamamoto A, Miyazaki T, Koshiyama H, Oda H, Namakura K, Tanaka S: Involvement of receptor activator of nuclear factor kappab ligand/osteoclast differentiation factor in osteoclastogenesis from synovocytes in rheumatoid arthritis. Arthritis Rheum 2000, 43:259-269.
20. Honigwood NJ, Kartoglou V, Chiu DJ, Roques E, Martin TJ, Gillespie MT: Activated T lymphocytes support osteoclast formation in vitro. Biochem Biophys Res Commun 1999, 265:144-150.
21. Redlich K, Hayer S, Maier A, Dunstan CR, Tohidast-Akrad M, Lang S, Turk B, Pietzschman P, Woloszczuk W, Harlambous S, et al.: Tumor necrosis factor alpha-mediated joint destruction is inhibited by targeting osteoclasts with osteoprotegerin. Arthritis Rheum 2002, 46:789-792.
22. Schett G, Middleton S, Bolon B, Stolina M, Brown H, Zuh L, Preto- torius J, Zach DJ, Kostenuk P, Feige U: Additive bone-protective effects of anabolic treatment when used in conjunction with RANKL and tumor necrosis factor inhibition in two rat arthritis models. Arthritis Rheum 2005, 52:1604-1611.
23. Lubberts E, Van Den Besselaar L, Oppers-Walgreen B, Schwarzenberger P, Coenen-de Roo CJ, Kollas G, Joosten LA, van Den Berg WB: IL-17 promotes bone erosion in murine collagen-induced arthritis.
arthritis through loss of the receptor activator of NF-kappa B ligand/osteoprotegerin balance. *J Immunol* 2003, 170:2655-2662.

29. Romas E, Sims NA, Hards DK, Lindsay M, Quinn JW, Ryan PF, Dunstan CR, Martin TJ, Gillespie MT: Osteoprotegerin reduces osteoclast numbers and prevents bone erosion in collagen-induced arthritis. *Am J Pathol* 2002, 161:1419-1427.

30. Lam J, Ross FP, Teitelbaum SL: RANK ligand stimulates anabolic bone formation. *J Bone Miner Res* 2001, 16(Suppl 1):1053.

31. Martin TJ, Sims NA: Osteoclast-derived activity in the coupling of bone formation to resorption. *Trends Mol Med* 2005, 11:78-81.

32. Karsdal MA, Henriksen K, Sorensen MG, Gram J, Schaller S, Dzieregiel MH, Heegaard AM, Christoffersen P, Martin TJ, Christiansen C, Bollerslev J: Acidification of the osteoclastic resorption compartment provides insight into the coupling of bone formation to bone resorption. *Am J Pathol* 2005, 166:467-476.