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Dickkopf-1 is a master regulator of joint remodeling

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Degenerative and inflammatory joint diseases lead to a destruction of the joint architecture. Whereas degenerative osteoarthritis results in the formation of new bone, rheumatoid arthritis leads to bone resorption. The molecular basis of these different patterns of joint disease is unknown. By inhibiting Dickkopf-1 (DKK-1), a regulatory molecule of the Wnt pathway, we were able to reverse the bone-destructive pattern of a mouse model of rheumatoid arthritis to the bone-forming pattern of osteoarthritis. In this way, no overall bone erosion resulted, although bony nodules, so-called osteophytes, did form. We identified tumor necrosis factor-α (TNF) as a key inducer of DKK-1 in the mouse inflammatory arthritis model and in human rheumatoid arthritis. These results suggest that the Wnt pathway is a key regulator of joint remodeling.

Affliction of joints is the hallmark of rheumatic disease. In addition to pain, both degenerative and inflammatory rheumatic diseases lead to a profound remodeling of the joint architecture, which causes functional disability and progressive crippling. This structural damage is largely responsible for the high socioeconomic burden of rheumatic disease, and definition of its molecular mechanism is therefore of key interest[1].

There are two major patterns of joint pathology in rheumatic diseases. One of these is progressive bone and joint destruction leading to joint instability. This pattern is the hallmark of rheumatoid arthritis and results in progressive joint deformity[2]. Notably, joints affected by rheumatoid arthritis usually lack signs of repair, which contributes to rapid loss of joint structure[2]. In contrast, diseases such as ankylosing spondylitis and psoriatic arthritis are very different from rheumatoid arthritis and represent the second pattern of joint pathology, which is characterized by new bone formation. After initial destructive changes, joints 'respond' by forming osteophytes, which are bony appositions originating from the juxta-articular periosteal lining[3]. These bone appositions are formed via endochondral ossification and can even bridge the entire joint cavity, resulting in immobilization of the affected joint. Local apposition of bone is also a hallmark of osteoarthritis, where osteophytes form the structural basis of well-known lesions such as Heberden nodules of the finger joints.

The molecular basis for these different arthritic patterns is unknown. In rheumatoid arthritis, the cytokine tumor necrosis factor (TNF) contributes substantially to the pathology of the disease, which is highlighted by the responsiveness of the disease toward TNF-blocking reagents[4,5]. TNF promotes destruction of bone by increasing the number of bone-resorbing osteoclasts and decreasing the number of bone-forming osteoblasts, thereby leading to an overall bias toward bone resorption[6,7]. In contrast, the structural joint pathology found in osteoarthritis or certain inflammatory joint diseases such as ankylosing spondylitis is completely different: initial erosive changes are followed by marked anabolic skeletal response, which results in the formation of osteophytes. It can thus be postulated that the structural differences in various joint diseases originate from master regulatory players of bone turnover, which allow differential remodeling of joints.

Overexpression of TNF in rodents largely mimics the changes of joint architecture found in human rheumatoid arthritis[8]. Increased TNF levels in joints lead to chronic inflammation, which results in progressive bone erosion. In humans with rheumatoid arthritis and in mice overexpressing TNF, invasion of inflammatory tissue into bone starts from periosteal sites and preferentially affects subchondral bone next to the joint space. These lesions emerge from an accumulation of osteoclasts, which resorb bone and subsequently allow the invasion of inflammatory tissue; this highlights the imbalance of bone turnover, where local bone resorption by far outweighs bone formation[9]. This imbalance is engendered by key molecules that regulate osteoclast differentiation, such as receptor activator of NF-kB ligand (RANKL), which are induced upon arthritis and mediate osteoclast-mediated destruction of the joint architecture[10]. Disruption of osteoclast activity, by genetic deletion or pharmacological blockade of essential differentiation molecules, completely abolishes arthritic bone erosion and preserves the joint architecture[10–12]. Notably, mechanisms attempting to counter-regulate arthritic bone destruction, such as increased bone formation, are missing in TNF-overexpressing mice and in humans with rheumatoid arthritis, whereas they are a hallmark of diseases such as ankylosing spondylitis or osteoarthritis.

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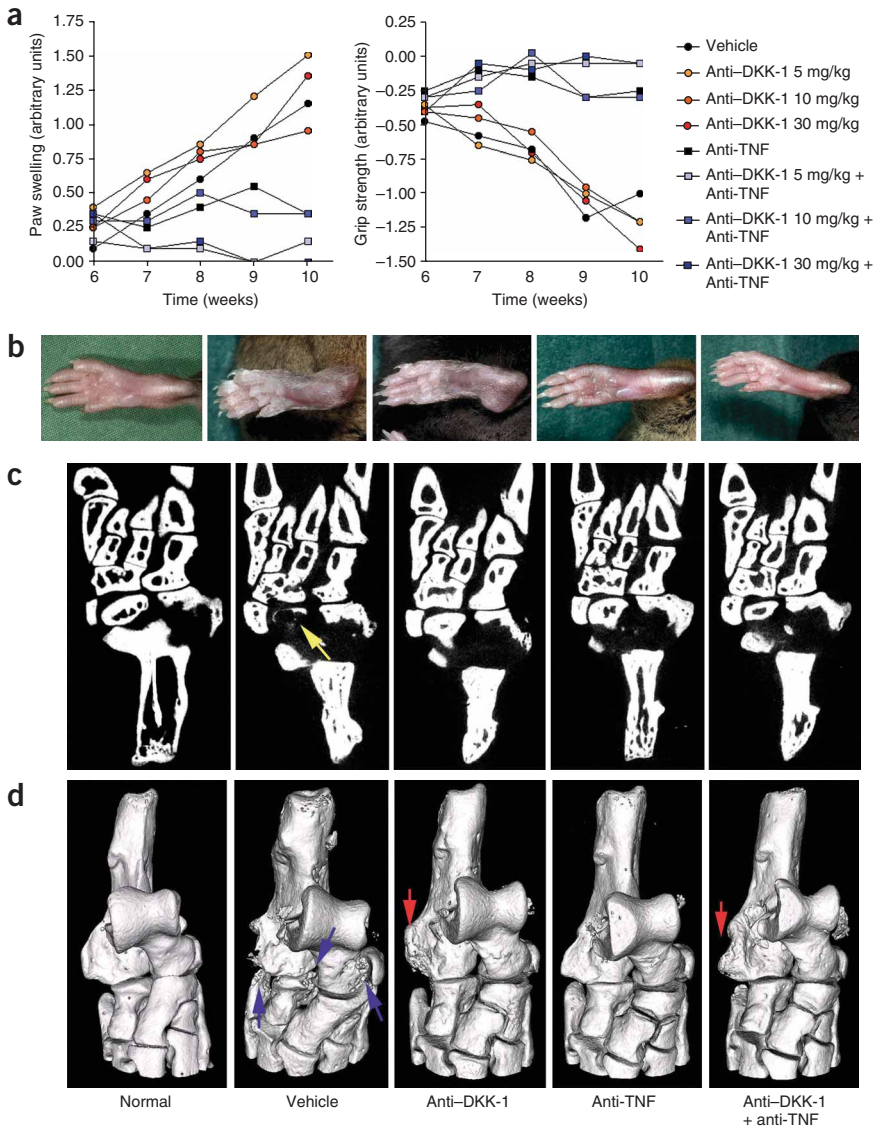

Figure 1 DKK-1 is not relevant for the inflammatory signs of arthritis but affects the skeletal shape of joints. (**a**) Effect of treatment of hTNFtg mice by vehicle, anti-TNF antibody, anti–DKK-1 antibody (various dosages) or a combination of both antibodies on paw swelling and grip strength over time (weeks 5–10). (**b**) Images of hind paws, "from left to right", of wild-type mice and hTNFtg mice treated with vehicle, anti–DKK-1 antibody (30 mg/kg), anti-TNF antibody (10 mg/kg) and a combination of these antibodies at week 10. (**c,d**) Microcomputed tomography slice images and three-dimensional surface rendering of the tarsal bones of wild-type mice and hTNFtg mice treated with vehicle, anti–DKK-1 antibody (30 mg/kg), anti-TNF antibody (10 mg/kg), and a combination of both antibodies. Yellow (in **c**) and purple (in **d**) arrows mark bone erosion; red arrows (in **d**) mark osteophytes.

We thus hypothesized that joint pathology affects regulators of bone formation, which drive either bone resorption or formation of new bone in the diseased joint. Proteins synthesized by the group of wingless (*Wnt*) genes are key mediators of osteoblastogenesis and govern the formation of the skeleton during the development of the embryo[13]. Several members of the Wnt protein family bind a receptor complex (consisting of LPR5/6 and frizzled receptors) on the plasma membrane of mesenchymal cells, which signals osteoblast differentiation by engaging the intracellular protein β-catenin[14,15]. Wnt signaling is modulated by several different families of secreted negative regulators. Among these, Dickkopf (DKK) is a family of cysteine-rich proteins comprising at least four different forms (DKK-1, DKK-2, DKK-3 and DKK-4). The best studied of these is DKK-1, which functions as a natural inhibitor of Wnt signaling[16,17]. When DKK-1 binds to the LPR5/6 receptor and a cell surface coreceptor, Kremen-1/2, it promotes internalization of the receptor complex and dampens the Wnt signal. Deletion of a single allele of DKK-1 increases bone mass in mice[18]. Recently, aberrant expression of DKK-1 in myeloma cells was shown to be associated with increased bone erosions in human multiple myeloma[19]. Thus, expression of DKK-1 in inflammatory and degenerative joint diseases may inhibit bone formation within the joint, further biasing the imbalance toward bone resorption.

RESULTS

DKK-1 blocks bone formation in the joint

In order to define a potential role of canonical Wnt signaling in arthritic joint remodeling, we used a neutralizing rat monoclonal antibody to mouse DKK-1 (anti–DKK-1) in a transgenic mouse model of rheumatoid arthritis. Considering that Wnt signaling might trigger new bone formation in joints, including the generation of osteophytes, we hypothesized that the level of DKK-1 is critical for determining whether a diseased joint undergoes destruction or reacts by forming new bone. Treatment with increasing doses of anti–DKK-1 antibody did not alter clinical signs of inflammation in human TNF transgenic (hTNFtg) mice (**Fig. 1a,b**). Radiographic and histopathological examination of joints revealed almost complete abolishment of inflammatory bone erosions (**Fig. 1c,d** and **Fig. 2a–d**) despite full-blown inflammation. This blockade of bone erosion appeared to be due to decreased osteoclast formation in the affected joints (**Fig. 2e**). To test our hypothesis in other animal models we performed additional analyses in collagen-induced arthritis (CIA) and glucose-6-phosphate isomerase (GPI)-induced arthritis, which revealed virtually identical results. In both CIA and GPI-induced arthritis, challenge with anti–DKK-1 antibody led to a protection of joints from structural damage and inhibited osteoclast formation (**Supplementary Fig. 1** online). Together, these data indicate that DKK-1 is a major contributor to bone loss in inflammatory joint disease. Prevention of bone loss was achieved despite active histopathological signs of inflammation, indicating a complete uncoupling of inflammation and bone loss.

Notably, blockade of DKK-1 activity in hTNFtg mice led to osteophyte formation within the inflamed joint at sites prone to structural damage (**Fig. 1c** and **Fig. 2a,b,f**). In contrast, osteophyte formation was consistently absent in untreated hTNFtg mice, suggesting that overexpression of TNF inactivates molecules involved in

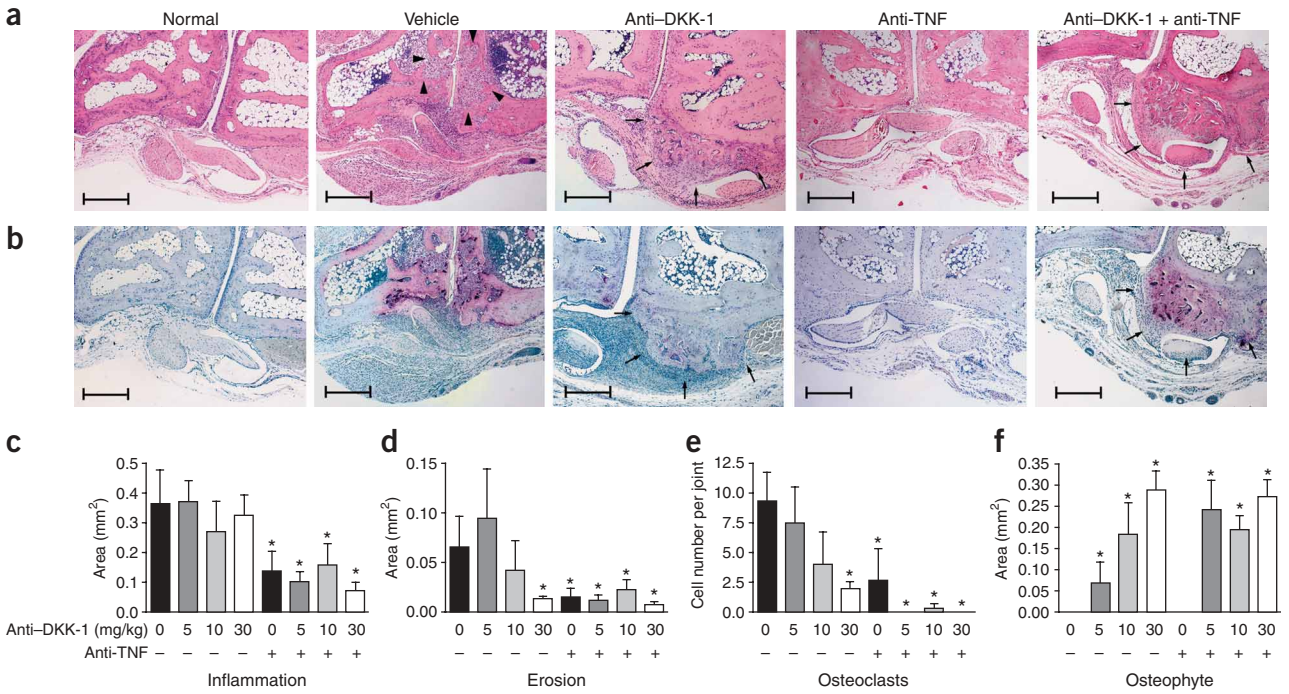

Figure 2 Inhibition of DKK-1 blocks bone erosion and promotes osteophyte formation (**a**) Microphotographs of H&E-stained tissue sections of the joint between the calcaneus and tarsal bones in wild-type mice and hTNFtg mice treated with vehicle, anti–DKK-1 antibody (30mg/kg), anti-TNF antibody (10 mg/kg) and a combination of these antibodies at week 10. (**b**) TRAP-stained tissue sections of the same area labeling osteoclasts as purple spots. Black arrowheads mark bone erosions; black arrows show the osteophyte. Scale bar, 40 μm. (**c**–**f**) Quantitative histomorphometric assessment of synovial inflammation, bone erosion, osteoclast numbers and osteophytes in tarsal joints.

osteophyte formation. Moreover, when we used specific antibodies to simultaneously block TNF and DKK-1, osteophytes formed at the same location despite the absence of major arthritic changes, suggesting that DKK-1 can regulate osteophyte formation in the absence of inflammatory tissue. The appearance of osteophytes upon blockade of DKK-1 identifies Wnt signaling as a key trigger in the formation of osteophytes. This concept is also fostered by consistent results in the two other models of inflammatory arthritis, CIA and GPI-induced arthritis that we tested, which showed rapid emergence of osteophytes upon blockade of DKK-1 (**Supplementary Fig. 1**).

Osteophytes are formed by endochondral ossification, which is accomplished by differentiation of periosteal cells to chondroblasts and osteoblasts. We labeled proteoglycans, which revealed areas of chondrogenic differentiation within osteophytes (**Fig. 3a** and **Supplementary Fig. 1**). We then examined undecalcified joint sections and found that these structures were predominantly composed of calcified tissue with metabolically active osteoblasts, osteoid seams and signs of mineralization (**Fig. 3b**). When we performed histomorphometrical examination of juxta-articular bone we further found an accumulation of osteoblasts, enhanced osteoid deposition and increased bone formation following blockade of DKK-1 (**Fig. 3c**). These findings show that DKK-1 not only promotes bone resorption but also effectively blocks bone formation and repair in the diseased joint. This notion was further supported by increased osteocalcin expression in joints (**Fig. 3b**) and elevated osteocalcin levels in the serum (**Supplementary Fig. 2** online). In contrast, osteoclast formation was strongly suppressed, as indicated by the paucity of osteoclasts in the joint and the low serum levels of bone degradation products, such as collagen type I crosslaps (**Supplementary Fig. 2**).

Inflammation induced the expression of DKK-1

DKK-1 obviously prevents osteophyte formation in arthritis by neutralizing anabolic repair mechanisms while supporting catabolic pathways of joint destruction. Transforming growth factor (TGF)-β and bone morphogenic proteins (BMP) are examples of anabolic molecules involved in joint remodeling[20,21], but, apparently, functional Wnt signaling is an essential step in this process. We therefore investigated the mechanisms that regulate the expression of DKK-1 in arthritis. The serum level of DKK-1 was consistently more than three times higher in hTNFtg mice than in wild-type mice (**Fig. 4a**). A similar elevation of systemic DKK-1 levels was also found in early CIA and in GPI-induced arthritis. However, in these models, DKK-1 levels dropped even below wild-type levels in the later stages of disease. This is in accordance with the later osteophytic response in these two models of arthritis, which is never found in hTNFtg mice. Moreover, DKK-1 expression was also increased in the synovial tissue of hTNFtg mice (**Fig. 4b**), while immunohistochemical investigations localized DKK-1 expression to fibroblast-like synovial cells as well as to neighboring chondrocytes (**Fig. 4c**). Our analyses in CIA further confirmed the increased local expression of DKK-1 in synovial tissue, showing a more than five-fold increase in arthritic as compared to normal mice. This upregulation was comparable to the levels of proinflammatory cytokines such as interleukin (IL)-1, IL-6, IL-8, macrophage chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1a and granulocyte colony-stimulating factor (G-CSF) or RANKL (**Supplementary Fig. 2**). The expression of the regulatory coreceptor of DKK-1, Kremen-1, which probably potentiates the effects of DKK-1, was also highly increased in TNF-mediated joint disease (**Fig. 4b**). In contrast, expression of Wnt proteins Wnt-4 and Wnt-5a was only slightly increased in arthritic mice, suggesting

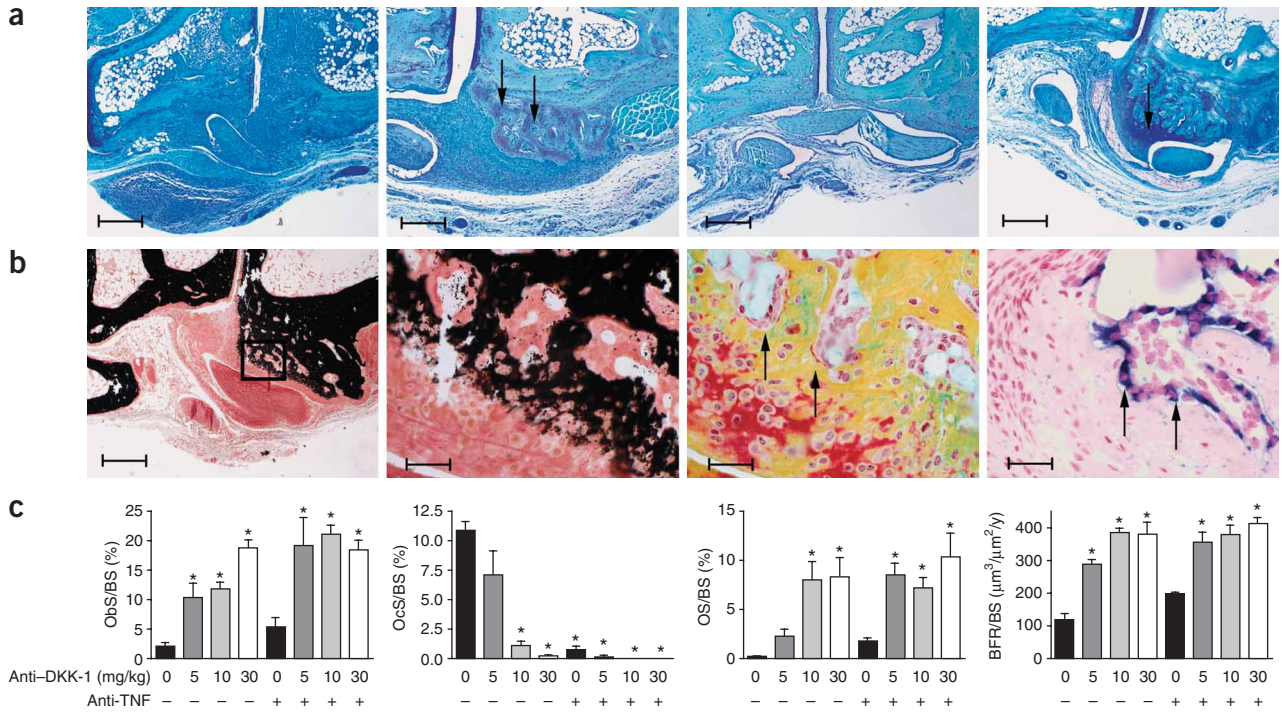

Figure 3 New bone formation next to inflamed joints is increased upon blockade of DKK-1. (**a**) Microphotographs of toluidine blue–stained joint sections of, "from left to right", hTNFtg mice treated with vehicle, anti–DKK-1 antibody (30 mg/kg), anti-TNF antibody (10 mg/kg) and a combination of these antibodies at week 10. Black arrows indicate areas of proteoglycan deposition. Scale bar, 40 μm. (**b**) Plastic sections of the tarsal joints after treatment with 30mg/kg anti–DKK-1 antibody. Microphotographs show von Kossa staining (first and second from left), Movat staining (second from right, black arrows) and *in situ* hybridization for osteocalcin (right, black arrow). Scale bars, 40 μm (left image) and 160 μm. (**c**) Dynamic bone histomorphometry of the tarsal bones of mice treated with vehicle, anti-TNF antibody, anti–DKK-1 antibody (various dosages) or a combination of both antibodies. Osteoblast-covered surface per bone surface (ObS/BS), osteoclast-covered surface per bone surface (OcS/BS), osteoid surface per bone surface (OS/BS) and bone formation rate per bone surface (BFR/BS) are given.

that functional Wnt signaling is effectively blocked by the marked upregulation of its inhibitors (**Fig. 4b**). Wnt-4 expression mostly occurred in synovial lining cells, whereas Wnt-5a was found in the synovial tissue within bone erosions (**Fig. 4c**).

We next addressed the issue of how DKK-1 is molecularly regulated in cultivated articular mesenchymal cells challenged by TNF. Namely, upregulation of DKK-1 expression involved TNF receptor-1 (TNFR1) as well as activation of p38 mitogen activated protein kinase (MAPK), as both the absence of TNFR1 (but not TNFR2) and the blockade of p38MAPK (but not PI3K or ERK) activation blocked the induction of DKK-1 (**Fig. 4d**). This mechanism was also supported by data showing the normalization of DKK-1 serum levels after systemic blockade of TNF or p38MAPK activation in arthritic mice (**Supplementary Fig. 2**). To gain further insight into the regulation of DKK-1 by TNF, we blocked p38MAPK signaling by soluble inhibitors and small interfering RNA. We inhibited p38MAPK by using the synthetic inhibitor SB203580 or p38-siRNA and saw effective blockade of downstream activation of MAPK-activated protein kinase-2 (MAPKAP2), as well as the induction of DKK-1 (**Fig. 4e**). In addition, when we used siRNA to inhibit kinases upstream of p38MAPK, we further showed that TNF induces DKK-1 via mitogen-activated protein kinase kinase-3 (MKK-3) but not MKK6 (**Fig. 4d**).

Cross-talk between Wnt and RANKL pathways in the joint

The marked effect of DKK-1 on joint architecture led us to hypothesize a tight interaction between DKK-1 and key molecules of joint degradation, such as the RANKL-osteoprotegerin (OPG) system. Targeting of DKK-1 by specific antibodies neutralized articular expression of DKK-1 in wild-type and *Dkk1* transgenic mice (*Dkk1*tg) (**Supplementary Fig. 2**). This blockade of DKK-1 changed a catabolic pattern of disease into an anabolic one, suggesting a wide interference of DKK-1 with bone formation and bone resorption. The antiresorptive potential of DKK-1 is likely to be based on an increased expression of OPG, a protein that interferes with RANKL-mediated osteoclast formation and bone resorption (ref. 10 and **Supplementary Fig. 2**). This cross-talk between DKK-1 and OPG was also found in wild-type mice and *Dkk1*tg mice, where DKK-1 inhibition was followed by elevated systemic OPG levels (**Supplementary Fig. 2**). This suggests that OPG expression is modulated by DKK-1. Furthermore, analysis of *Tnfsf11b* (*Opg*) mutants showed unchanged DKK-1 levels in *Tnfsf11b*$^{-/-}$ mice but high DKK-1 levels in *Tnfsf11b*tg mice, supporting the notion that OPG regulates DKK-1 expression through a feedback loop (**Supplementary Fig. 2**). This cross-talk between DKK-1 and OPG suggests that at least the antiresorptive feature of DKK-1 inhibition is caused by increased OPG expression. Indeed, when we inhibited OPG by intra-articular injection of OPG-siRNA into mice challenged for CIA and treated with anti–DKK-1, we observed an uncoupling of anabolic and catabolic effects (**Supplementary Fig. 3** online). Whereas osteophyte formation was not affected, bone resorption by osteoclasts re-emerged, which clearly supports the concept that the antiresorptive property of DKK-1 is mediated through OPG. In contrast, addition of recombinant OPG abrogated osteoclast formation in CIA but did not change osteophyte formation (**Supplementary Fig. 3**). This latter finding shows that

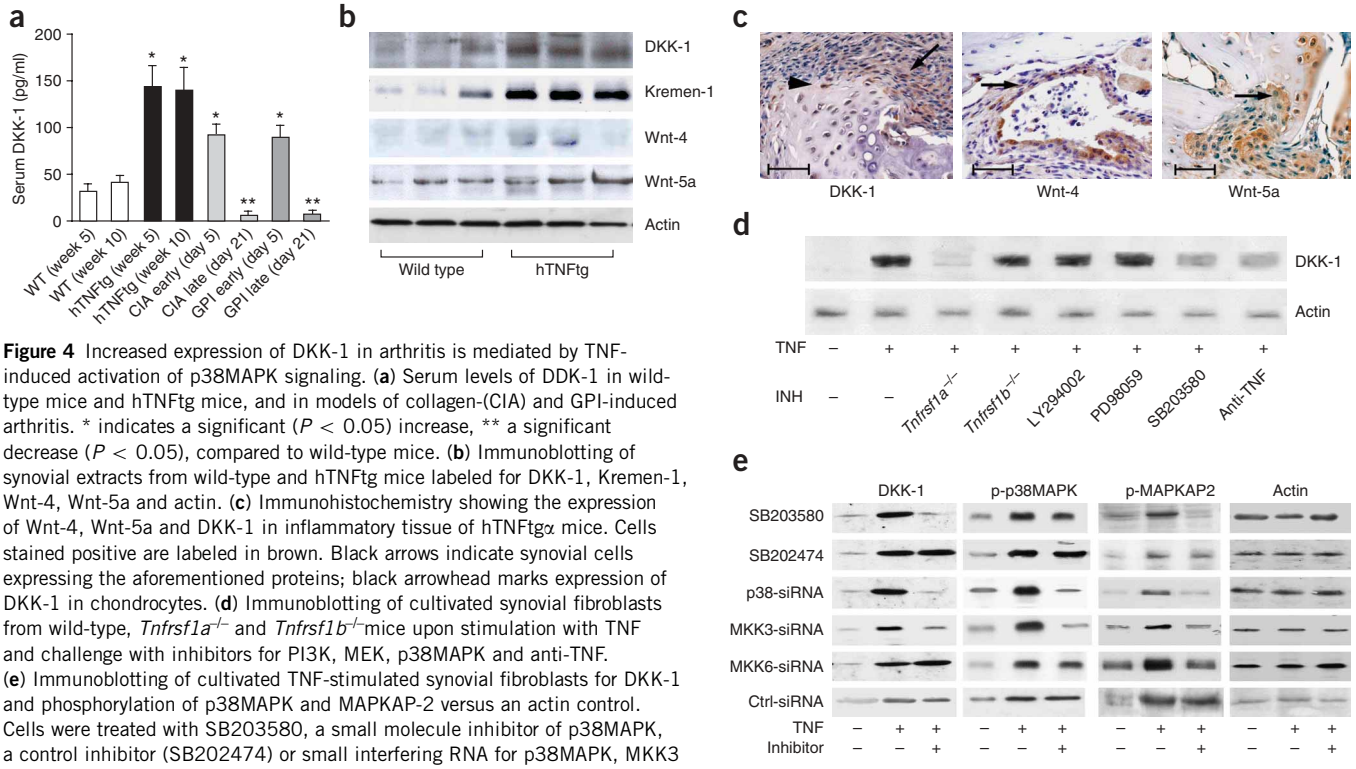

Figure 4 Increased expression of DKK-1 in arthritis is mediated by TNF-induced activation of p38MAPK signaling. (**a**) Serum levels of DDK-1 in wild-type mice and hTNFtg mice, and in models of collagen-(CIA) and GPI-induced arthritis. * indicates a significant ($P < 0.05$) increase, ** a significant decrease ($P < 0.05$), compared to wild-type mice. (**b**) Immunoblotting of synovial extracts from wild-type and hTNFtg mice labeled for DKK-1, Kremen-1, Wnt-4, Wnt-5a and actin. (**c**) Immunohistochemistry showing the expression of Wnt-4, Wnt-5a and DKK-1 in inflammatory tissue of hTNFtgα mice. Cells stained positive are labeled in brown. Black arrows indicate synovial cells expressing the aforementioned proteins; black arrowhead marks expression of DKK-1 in chondrocytes. (**d**) Immunoblotting of cultivated synovial fibroblasts from wild-type, *Tnfrsf1a*$^{-/-}$ and *Tnfrsf1b*$^{-/-}$mice upon stimulation with TNF and challenge with inhibitors for PI3K, MEK, p38MAPK and anti-TNF. (**e**) Immunoblotting of cultivated TNF-stimulated synovial fibroblasts for DKK-1 and phosphorylation of p38MAPK and MAPKAP-2 versus an actin control. Cells were treated with SB203580, a small molecule inhibitor of p38MAPK, a control inhibitor (SB202474) or small interfering RNA for p38MAPK, MKK3 and MKK6.

osteophytes can form independent from RANKL-OPG and involves bone anabolic pathways modulated by DKK-1.

We thus questioned whether blockade of DKK-1 restores functional Wnt signaling in the joints of arthritic mice. Immunoblot analyses of synovial tissue revealed that blockade of DKK-1 effectively decreases *in vivo* phosphorylation of β-catenin (**Supplementary Fig. 4** online). Notably, nonphosphorylated β-catenin can escape ubiquitination and degradation and leads to the downstream activation of the canonical Wnt signaling pathway[22]. Accordingly, nuclear localization of β-catenin in the synovial tissue was markedly increased and the strongest nuclear accumulation was found in synovial fibroblast adjacent to osteophytes (**Supplementary Fig. 4**). Moreover, expression of conductin (also called axin-2), a component of the Wnt-signaling pathway, which is activated by functional Wnt signaling[23] was strongly unregulated in the joints after blockade of DKK-1 (**Supplementary Fig. 4**). Functional assays on cultured calcaneal bones revealed that TNF inhibits anabolic periosteal responses to BMP-2 and Wnt-3a, and that this inhibitory effect could be reversed upon blockade of DKK-1 (**Supplementary Fig. 4**). These data show that DKK-1 effectively blocks the anabolic function of Wnt proteins and that the neutralization of DKK-1 relieves anabolic pathways from the suppressive effects of TNF.

Differential regulation of DKK-1 in human joint disease

Our next aim was to investigate the role of DKK-1 in human joint disease. DKK-1 expression was increased in joint sections from rheumatoid arthritis subjects undergoing joint replacement surgery, as compared to osteoarthritis subjects (**Fig. 5a**). In the rheumatoid arthritis subjects, DKK-1 was widely expressed in the inflamed synovium, especially in fibroblast-like synoviocytes embedded in the inflamed tissue, in synovial microvessels and in cartilage adjacent to inflammatory tissue (**Fig. 5b**). In contrast, these subjects had only limited expression of β-catenin in the joints and only a few spots at the interface between synovial inflammatory tissue and bone showed nuclear expression of β-catenin (**Fig. 5c**). We then assessed serum levels of DKK-1 by an enzyme-linked immunosorbent assay measuring the functional binding of serum DKK-1 to its receptor LRP-6. Serum levels of DKK-1 were approximately twice as high in subjects with rheumatoid arthritis (mean ± s.e.m. 31.5 ± 1.9 pg/ml) compared to healthy controls (17.0 ± 0.7 pg/ml) and were closely related to clinical disease activity (Spearman's rho for correlation: DKK-1 versus disease activity score (DAS) 28, 0.75) (**Fig. 5d**). In contrast, DKK-1 levels in ankylosing spondylitis, an inflammatory joint disease with high prevalence of osteophytes, were very low and even below the levels in healthy subjects (5.8 ± 0.3 pg/ml), suggesting a role of DKK-1 in differential remodeling of human joint architecture. There was no relation of DKK-1 to disease activity in ankylosing spondylitis (Spearman's rho for correlation: DKK-1 versus Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) score, –0.07) (**Fig. 5d**). Moreover, systemic inhibition of TNF significantly ($P < 0.01$) reduced elevated DKK-1 levels to almost the normal range in subjects with rheumatoid arthritis (18.0 ± 1.2 pg/ml), suggesting that TNF is a driving force for the upregulation of DKK-1 in human arthritis (**Fig. 5d**).

DISCUSSION

Here we show that DKK-1 plays a key role in the remodeling of joints by two mechanisms. First, DKK-1 impairs local bone formation, which is particularly deleterious in rheumatoid arthritis, where bone is rapidly degraded by an osteoclast-forming inflammatory tissue (**Fig. 6**). This insight fosters the role of local bone formation and repair in inflammatory joint disease. The upregulation of DKK-1 by TNF in arthritis, the high efficacy of DKK-1 inhibition in affecting joint structure and the return to baseline of increased DKK-1 levels

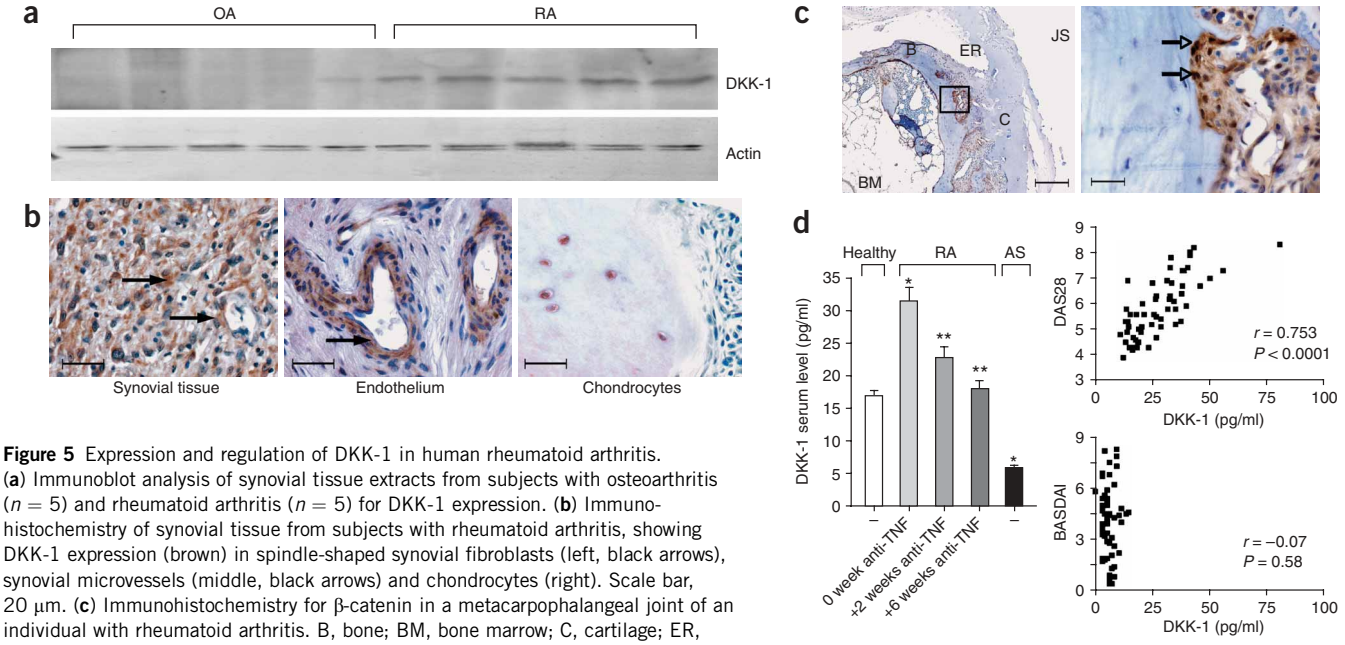

Figure 5 Expression and regulation of DKK-1 in human rheumatoid arthritis. (**a**) Immunoblot analysis of synovial tissue extracts from subjects with osteoarthritis ($n = 5$) and rheumatoid arthritis ($n = 5$) for DKK-1 expression. (**b**) Immunohistochemistry of synovial tissue from subjects with rheumatoid arthritis, showing DKK-1 expression (brown) in spindle-shaped synovial fibroblasts (left, black arrows), synovial microvessels (middle, black arrows) and chondrocytes (right). Scale bar, 20 μm. (**c**) Immunohistochemistry for β-catenin in a metacarpophalangeal joint of an individual with rheumatoid arthritis. B, bone; BM, bone marrow; C, cartilage; ER, erosion; JS, joint space. Arrows show nuclear staining of β-catenin. Right image is an enlarged detail of the left image. Scale bars, 160 μm (left) and 20 μm (right). (**d**) Measurement of DKK-1 serum levels in healthy controls, subjects with rheumatoid arthritis (RA) before and 2 and 4 weeks after initiation of TNF blockade, and subjects with ankylosing spondylitis (AS). Values are means ± s.e.m. * indicates significant ($P < 0.05$) difference versus healthy controls; ** indicates significant ($P < 0.01$) difference versus rheumatoid arthritis subjects before treatment. Scatter plots show the correlation of DKK-1 level with disease activity in rheumatoid arthritis subjects (DAS28 score) and ankylosing spondylitis subjects (BASDAI score).

upon TNF blockade in human disease suggest that at least part of the destructive effect of TNF on joints is mediated by DKK-1. On the other hand, low levels of DKK-1 appear to be crucial for the emergence of osteophytes, which suggests that Wnt signaling is a key trigger for bone formation in the joint. Local bone formation in the form of osteophyte formation is absent in rheumatoid arthritis but is a hallmark of inflammatory and degenerative joint diseases such as ankylosing spondylitis and osteoarthritis, respectively (**Fig. 6b,c**). Second, blockade of DKK-1 also interfered with local bone resorption by reducing osteoclast numbers in the joints (**Fig. 6b,c**). The impact on local bone resorption is based on the regulation of OPG expression by canonical Wnt signaling, which is illustrated by increased serum levels of OPG upon DKK-1 inhibition[24].

The regulation of systemic bone mass by means of Wnt signaling is of growing scientific interest. The Wnt coreceptor LRP5 is a critical regulator of bone mass[15]. Thus activation of LRP5 affects bone accrual during growth and regulates the establishment of peak bone mass[25]. In particular, the Wnt protein family, which engages the LRP5/6 receptors on mesenchymal cells, drives new bone formation and leads to an increase in systemic bone mass. The Wnt pathway is therefore considered an interesting target for bone anabolic therapies. DKK-1 is a Wnt antagonist that cross-links LRP5/6 with Kremen-1 and prevents its activation by Wnt. Recent data suggest that DKK-1 expression seems to be critical for systemic bone mass, as heterozygous DKK-1–deficient mice show increased bone formation and bone mass[18]. On the other hand, overexpression of DKK-1 in mesenchymal

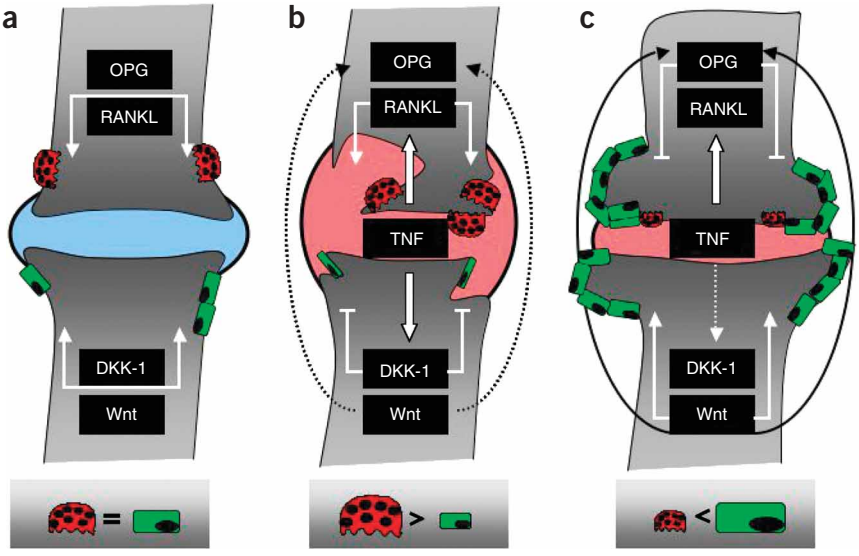

Figure 6 DKK-1 is critical for joint remodeling. (**a**) In a physiological state, cortical bone formation and resorption next to joints are in balance. (**b**) Inflammatory arthritis such as rheumatoid arthritis leads to an imbalance between bone formation and resorption. Bone formation is hampered by TNF-mediated expression of DKK-1, which suppresses Wnt signals, whereas bone resorption is enhanced by expression of RANKL. (**c**) Blockade of DKK-1 relieves Wnt signaling from DKK-1–mediated suppression and induces bone formation mirrored by the growth of osteophytes. Moreover, Wnt proteins induce OPG expression, which blocks RANKL-mediated bone resorption.

cells impairs their final differentiation into osteoblasts as well as osteoblast function[18]. Our data extend these genetic approaches and suggest that inflammation is a major pathophysiological inducer of DKK-1 expression, which provides a new link between the immune system and bone formation. Cytokines such as TNF induce DKK-1 and impair bone formation during inflammation, which is reflected by the severe destruction of the joint architecture during arthritis.

Our data also point to an important cross-talk between the bone anabolic Wnt and the bone catabolic RANKL pathway. Although the heterozygous deletion of DKK-1 does not directly influence bone resorption[18], our disease models indicate that the balance between Wnt and DKK-1 is also critical for bone resorption. Blockade of DKK-1 leads to a profound inhibition of osteoclast formation and bone resorption in joints, suggesting that the cross-talk between the two systems is of biological relevance. Molecularly, OPG, a key component of the RANKL-RANK system, seems to be regulated by the Wnt system and vice versa, which is also backed by recent findings[24]. We show a mutual inverse regulation of OPG and DKK-1, which allows DKK-1 to have at least an indirect influence on osteoclastogenesis and bone resorption. This dual action identifies DKK-1 as key regulator of pathologic joint remodeling, which is based on the interplay between anabolic and catabolic pathways. Thus, joint destruction can never be seen in isolation from the mechanisms of remodeling and repair, which is highlighted by the vastly different phenotypes of joint diseases: a bone-destructive phenotype entailing joint instability in the case of rheumatoid arthritis and a bone anabolic reaction pattern leading to joint ankylosis in osteoarthritis and ankylosing spondylitis. The molecular determinants responsible for these differences in joint remodeling were largely unknown thus far, but constitute attractive therapeutic targets for the modulation of joint architecture. These data suggest DKK-1 as a key candidate for this process.

METHODS

Animals and treatments. Human TNF transgenic (hTNFtg) mice (strain Tg197; C57BL/6) have been described previously[15]. CIA was induced in DBA1 mice by 100 μg chicken collagen type II (Sigma) in complete Freund adjuvant (CFA) on day 1 and collagen type II in incomplete Freund adjuvant on day 10. GPI-induced arthritis was initiated by 350 μg rabbit muscle glucose-phosphate-isomerase (GPI) in 200 μl CFA. Mice were treated with the following agents: vehicle (PBS), infliximab (chimeric antibody to TNF; 10 mg/kg three times a week intraperitoneally (i.p.); ref. 26), a rat antibody to mouse DKK-1 (5, 10 and 30 mg/kg three times a week i.p.), osteoprotegerin (3 times weekly with 10mg/kg i.p.; ref. 25) and OPG siRNA (Santa Cruz; 25μg in 0.05% atelocollagen intraarticularly; ref. 27). All procedures were approved by the animal ethics committee of the Center of Biomedical Research of the Medical University of Vienna.

Patient characteristics. Rheumatoid arthritis subjects fulfilled the American College of Rheumatology (ACR) diagnostic criteria for rheumatoid arthritis, had a disease duration longer than 1 year and had active disease leading to TNF-blocker therapy (infliximab 5mg/kg) (ref. 28). Patients with ankylosing spondylitis were from the Outcome in Ankylosing Spondylitis International Study (OASIS) cohort and fulfilled the New York criteria for the diagnosis of disease[29]. Disease activity was assessed by DAS28 in rheumatoid arthritis subjects, and BASDAI in ankylosing spondylitis subjects[30,31]. Written informed consent was obtained from all subjects.

Assessment of arthritis. Paw swelling and grip strength were assessed by a semiquantitative score as described previously[12]. Microcomputed tomography of joints was performed by a desktop microcomputed tomography system (GE eXplore Locus SP Specimen Scanner; GE Healthcare). Histological analyses were performed on formalin-fixed, decalcified, paraffin-embedded tissue sections stained with hematoxylin and eosin (H&E), tartrate-resistant acid phosphatase (TRAP) and toluidine blue[12]. Synovial inflammation, bone erosions, osteoclast numbers and osteophytes were quantified by digital image analysis (OsteoMeasure, OsteoMetrics). Histomorphometry was performed on methacrylate-embedded undecalcified plastic sections stained with von Kossa for bone and Movat for osteoid.

Immunohistology and *in situ* hybridization. Deparaffinized ethanol-dehydrated tissue sections from hTNFtg mice ($N = 10$) and from synovectomy specimens from subjects with rheumatoid arthritis ($N = 10$) were pretreated with 0.05% protease XIV (Sigma) for staining of Wnt-5a, high temperature unmasking (20 min in citrate buffer, pH 6.0) for Wnt-4, or were left without pretreatment for DKK-1 (all R&D) and β-catenin (Sigma). We used a cDNA probe for the osteocalcin *in situ* hybridization, and *in situ* hybridization was performed as previously described[32].

Immunoblotting of mouse and human joints. Paws of wild-type and hTNFtg mice and synovial tissue from subjects with osteoarthritis and rheumatoid arthritis were snap-frozen in liquid nitrogen and mechanically homogenized in buffer containing 20 mM HEPES, 0.4 M NaCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 1 mM EDTA, 0.1 mM EGTA and 20% glycerol, as well as protease and phosphatase inhibitors (Sigma). Antibodies to the following proteins were used: actin (Sigma), phospho-β-catenin (Abcam) and β-catenin (R&D), conductin, DKK-1 (R&D), Kremen-1 (R&D), phospho-MAPKAP-2 (Biosource), phospho-p38MAPK and total p38MAPK (Santa Cruz), Wnt-4 and Wnt-5a (R&D).

Synovial cell culture and tissue cultures. Synovial cells was isolated from knee joints of wild-type, *Tnfrsf1a*$^{-/-}$ and *Tnfrsf1b*$^{-/-}$ mice, and challenged with 10 ng/ml mouse TNF (R&D) as well as inhibitors against PI3K (20 mM LY 294002), MEK/ERK (20 mM PD98059), p38MAPK (SB203580, 20 mM; all Calbiochem) and TNF (1 μg/ml infliximab). RNA interference of p38MAPK, MKK3 and MKK6 was performed by the respective siRNA/siAB assay kits (Upstate Biotechnologies). Explant cultures of calcaneal bones were cultivated with BMP-2 (10 ng/ml), Wnt-3a (100ng/ml), mouse TNF (10 ng/ml; all R&D) or anti–DKK-1 antibody (1 μg/ml) for 2 weeks, embedded in methacrylate and stained by Movat. Periosteal surface was then analyzed for the presence of osteoblasts as well as osteoid deposition.

Synovial cytokine quantification. Tibiotarsal joints were pulverized in liquid nitrogen and protein extracted with a standard digestion buffer (50 mM Tris buffer, pH 7.4, containing 0.1 M sodium chloride and 0.1% Triton X-100). Cytokine levels were determined by Luminex assay (Luminex Corporation).

Serum measurements. Serum levels of osteocalcin were measured by immunoradiometric assay (Immutopics), desoxypyridinolin (DPD) crosslaps by enzyme immunoassay (Quidel) and osteoprotegerin by enzyme immunoassay (Biomedica). Serum DKK-1 was measured by coating microtiter plates with 1 μg/ml human LRP-6 chimera (R&D) before addition of human serum samples. Detection was performed by biotinylated antibody to human DKK-1 immunoglobulin (1:200, R&D).

Statistical analysis. Data are given as mean ± s.e.m. Group mean values were compared by ANOVA.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

D.Diarra conducted the *in vivo* analyses of hTNFtg mice and contributed to manuscript preparation. M.S. performed the analyses of collagen-induced arthritis

and contributed to manuscript preparation. K.P. and J.Z. worked on the *in vitro* analysis of hTNFtg mice and human samples. M.S.O. performed microcomputed tomography. D. Dwyer conducted the analyses on collagen-induced arthritis. A.K. collected human samples and worked on *in vitro* analysis of hTNFtg mice. J.S. conducted data analyses on murine and human samples. M.H. and C.S. analyzed the GPI-induced arthritis model. D.v.d.H. and R.L. analyzed samples from spondylarthropathy patients. D.L., W.G.R. and G.S. supervised the project and contributed to manuscript preparation.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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