Published in final edited form as:

Ann N Y Acad Sci. 2016 July ; 1376(1): 53–64. doi:10.1111/nyas.13000.

Systemic neutralization of TGF- β attenuates osteoarthritis

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Abstract

Osteoarthritis (OA) is a major source of pain and disability worldwide with no effective medical therapy due to poor understanding of its pathogenesis. Transforming growth factor β (TGF- β) has been reported to play a role in subchondral bone pathology and articular cartilage degeneration during the progression of osteoarthritis. In this study, we demonstrated that systemic use of a TGF- β -neutralizing antibody (1D11) attenuates OA progression by targeting subchondral bone pathological features in rodent OA models. Systemic administration of 1D11 preserves the subchondral bone microarchitecture, preventing articular cartilage degeneration by inhibition of excessive TGF- β activity, both in subchondral bone and in the circulation. Moreover, the aberrant increases in the numbers of blood vessels, nestin⁺ mesenchymal stromal/stem cells (MSCs), and osterix⁺ osteoblast progenitors were normalized by 1D11 systemic injection. Thus, systemic neutralization of excessive TGF- β ligands effectively prevented OA progression in animal models, with promising clinical implications for OA treatment.

Keywords

osteoarthritis; subchondral bone; cartilage; TGF- β ; antibody

Introduction

Osteoarthritis (OA) is the most common degenerative joint disorder, affecting an estimated 26.9 million U.S. adults in 2005.¹ Clinical symptoms include joint pain with functional impairment that is progressive, eventually necessitating joint replacement.² The traditional view of OA as a disease mainly of the articular cartilage of synovial joints is gradually giving way to more complex interplay between the articular cartilage and the subchondral bone, with resultant cartilage degeneration, subchondral bone sclerosis and edema, inflammation, and osteophyte formation.^{3,4} In a healthy joint, the articular cartilage and subchondral bone act as a functional unit, with the cartilage serving as a primary point of

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Conflicts of interest

The authors declare no competing financial interests.

pressure impact that is transmitted to the subchondral bone to initiate a cascade of biochemical/metabolic activities (remodeling) essential for the maintenance of the integrity of the articular cartilage.^{5,6} Coupled bone remodeling, where osteoclast and osteoblast activity are temporally and spatially regulated, ensures the integrity of the subchondral bone.⁷ Specifically, osteoclasts resorb bone and generate a bone marrow microenvironment, which is followed by targeted migration and differentiation of MSCs to support osteogenesis and angiogenesis for subsequent osteoblast bone formation.⁸ Unstable mechanical loading, as occurs in ligament injury, excessive weight bearing, or muscle weakness with aging, results in changes in the subchondral bone and eventually the articular cartilage.⁶ Transforming growth factor β (TGF- β) plays an important role in the maintenance of homeostasis between the subchondral bone and the articular cartilage, specifically in the temporospatial regulation of osteoclastic and osteoblastic activity in the subchondral bone, as well as in chondrogenesis, including chondrogenic condensation and chondroprogenitor cell proliferation and differentiation in the articular cartilage.⁹ TGF- β inhibits terminal differentiation of chondrocytes, thereby blocking cartilage matrix calcification and vascularization to maintain ECM integrity.¹⁰ TGF- β seems to have a dual role in the pathogenesis of OA: a protective effect on the articular cartilage, increasing the synthesis of proteoglycans, and a deleterious effect on subchondral bone, causing sclerosis and osteophyte formation at joint margins. Inhibition of endogenous TGF- β during experimental OA prevents osteophyte formation but also impairs cartilage repair, resulting in cartilage degeneration.¹¹

Accumulating evidence indicates that high levels of active TGF- β in subchondral bone, as seen in acute injury, disrupt the normal homeostatic mechanism essential for cartilage and joint integrity.^{11,12} TGF- β was found to be aberrantly elevated in OA subchondral bone in both human specimens and various animal models.^{13,14} Alterations of the normal subchondral bone structure and degeneration of the articular cartilage were observed in transgenic mice with constitutive expression of active TGF- β by osteoblastic cells.¹⁴ Aberrant elevation of active TGF- β in subchondral bone is associated with early signs of OA, including bone marrow lesions.¹⁵ High levels of active TGF- β induce clustering of MSCs/osteoprogenitors in the subchondral bone marrow and the formation of marrow osteoid islets.¹⁶ Indeed, OA progression was attenuated in the anterior cruciate ligament transection (ACLT) mouse model when the TGF- β type II receptor was deleted in MSCs.^{14,17}

Following an acute injury, such as ACLT, there is increased osteoclast bone resorption.¹⁸ The subchondral bone marrow microenvironment changes substantially, resulting in woven bone and angiogenesis. We have previously found that excessive activation of TGF- β by increased osteoclast bone resorption uncouples bone resorption and formation, contributing to the sclerotic phenotype in the subchondral bone in OA animal models.¹⁴ High levels of TGF- β result in erroneous recruitment of MSCs and the formation of osteoid islets. Vascularization and innervation of articular cartilage have also been noted in OA,^{19,20} with blood vessels and nerves originating from subchondral bone and breaching the tidemark in the early stages. As such, targeting excessive TGF- β activation could lead to maintenance of the structural and functional integrity of the articular cartilage–subchondral bone unit and potentially slow the progression of OA. This modulation of TGF- β activity could be either

direct, through TGF- β inhibitor/antibody, or indirect, via PTH-induced modification of the microenvironment.²¹

In this study, we looked at the effect of systemic administration of TGF- β -neutralizing antibody on modifying the pathogenesis and progress of OA. The effects of TGF- β on articular cartilage can be regulated at different levels, including activation of matrix latent TGF- β and the expression of different receptors or their downstream intracellular signaling components. We used 1D11, a known antibody of TGF- β that targets TGF- β 1, - β 2, and - β 3,²² and found that targeted inhibition of TGF- β signaling with neutralizing antibody treatment attenuates the progression of OA by decreasing subchondral bone sclerosis and slowing the degeneration of articular cartilage in OA rodent models through reduction of uncoupled bone formation and angiogenesis.

Results

Systemic injection of TGF- β -neutralizing antibody attenuates OA progression in ACLT mice

To investigate the potential effects of systemic injection of TGF- β -neutralizing antibody on OA progression, we generated a destabilized osteoarthritis animal model by transection of the anterior cruciate ligament (ACL) of 3-month-old mice, as described previously.¹⁴ The TGF- β -neutralizing antibody 1D11 was administered intraperitoneally to mice following ACLT surgery. To determine the optimal dose of 1D11, we injected ACLT mice or sham-operated controls with different concentrations of 1D11 antibody at various intervals, as illustrated in Table 1. Hematoxylin and eosin (H&E) staining revealed that the thickness of the calcified cartilage in 1D11-treated ACLT mice was decreased compared to the vehicle-treated ACLT mice (Table 2). Similarly, proteoglycan loss in cartilage was observed at 60 days after surgery in the ACLT mice treated with control antibody injections, which was consistent with previous observations (Fig. 1A and B). Systemic 1D11 treatment slowed the degeneration of articular cartilage in the ACLT mice model (Fig. 1C). We further analyzed the effects of different dosages on the articular cartilage. We found that a low dose of 1 mg/kg administered either three times a week, once a week, or once a month had minimal effects in preventing articular cartilage degeneration after ACLT (Table 2 and Fig. 1C). Higher concentrations, starting at 3 mg/kg and administered at the same dosing intervals (three times a week, once a week, and once a month) showed some improvement in proteoglycan stabilization and cartilage protection. Next, we used 5 mg/kg at the same dosing intervals. We found much greater preservation of articular cartilage with the 5-mg/kg dose given three times a week or once a week. We also used 10 mg/kg given over the same time intervals as previously stated. Interestingly, we observed thinner hyaline cartilage and proteoglycan loss in articular cartilage using 10 mg/kg of 1D11. This shows that the level of TGF- β needed to maintain subchondral bone/articular cartilage integrity is tightly controlled. Osteoarthritis Research Society International (OARSI) scores reflect degeneration of articular cartilage.²³ The results revealed that injection of 5 mg/kg three times per week obtained the best efficacy in attenuation of OA progression relative to the other doses (Fig. 1D). Injection of 5 mg/kg three times per week also inhibited expression of

matrix metalloproteinase 13 (MMP-13) and collagen X (Col X), indicating protection from degeneration of articular cartilage²⁴⁻²⁶(Fig. 1E and F).

Inhibition of TGF- β activity by injection of neutralizing antibody prevents aberrant subchondral bone changes

We next examined whether the protective effects on articular cartilage are associated with inhibition of TGF- β activity in the subchondral bone, as high levels of active TGF- β induce uncoupled subchondral bone remodeling and lead to degeneration of articular cartilage. Analysis of tibial subchondral bone structure by micro computed tomography (micro-CT) showed that, at a low concentration (1 mg/kg), 1D11 had no effect on subchondral bone preservation, whereas higher concentrations, starting at 3 mg/kg, improved subchondral bone structure (Fig. 2A–C). The tibial subchondral bone volume (TV) in ACLT mice increased substantially relative to sham-operated controls after surgery. These changes in ACLT mice were normalized by antibody treatment (Fig. 2D). The decreased bone volume fraction (BV/TV) after ACLT also improved with antibody injection (Fig. 2E). The thickness of the subchondral bone plate (SBP) decreased significantly in the ACLT mice at 2 months after surgery. Treatment with 5 mg/kg or 10 mg/kg prevented this decrease in SBP, with dosing of three times a week resulting in better improvement than once a week (Fig. 2F). Moreover, trabecular pattern factor (Tb.Pf),²⁷ a measure of trabecular bone stability, was reduced by antibody treatment in the ACLT mice compared to vehicle-treated ACLT mice, indicating an improvement of the connectivity and microarchitecture of trabecular bone (Fig. 2G). Together, these results suggest that systemic administration of 1D11 at an optimal dose could protect the subchondral bone structure, preventing the degeneration of articular cartilage and thus slowing the development of osteoarthritis. Uncoupled subchondral bone remodeling could be prevented by systemic administration of TGF- β -neutralizing antibody.

Aberrant osteogenesis and vessel formation in subchondral bone was abrogated by injection of 1D11

To examine the mechanistic effect of 1D11 in subchondral bone on protection of the joints, sham controls and ACLT rodent models were treated with the optimal dosage of 5 mg/kg injected three times per week. Immunostaining of tibia sections showed that the increase in phosphorylated Smad2/3 (pSmad2/3)⁺ cells in the subchondral bone after ACLT was significantly decreased after treatment with 1D11, compared with vehicle-treated controls (Fig. 3A). Interestingly, pSmad2/3 levels in articular cartilage were reduced in ACLT mice, which is consistent with previous studies.¹¹ Systemic inhibition of TGF- β with 5 mg/kg of 1D11 resulted in less pronounced reduction in the levels of pSmad2/3 in the articular cartilage (Fig. 3B). Immunohistochemical staining for nestin, which is expressed primarily in adult bone marrow MSCs,^{28,29} showed that 1D11 significantly attenuated the increase in the number of nestin⁺ MSCs in the subchondral bone post-ACLT relative to the vehicle-treated group (Fig. 3C). There was no statistically significant difference between the numbers of nestin⁺ cells in the 1D11-treated group after ACLT and those for the sham controls. Furthermore, nestin⁺ MSCs resided close to the blood vessels in the sham group, whereas in the vehicle-treated ACLT mice, large amounts of nestin⁺ cells were dispersed throughout the bone marrow. This abnormal distribution was prevented by treatment with 1D11, with redistribution of nestin⁺ cells similar to that seen in the sham group. Similarly,

osterix⁺ osteoprogenitors, normally located close to the bone surface in sham-operated controls, were found in significantly higher clusters in the bone marrow of vehicle-treated ACLT mice. The aberrant increase and relocation into the bone marrow was attenuated by 1D11 treatment (Fig. 3D). Our previous observation indicated that high concentrations of active TGF- β after ACLT result in increased angiogenesis in the subchondral bone, which is a pathological change.³⁰ We therefore examined the potential effects of 1D11 treatment on angiogenesis in subchondral bone. The result showed that the number of CD31⁺ endothelial progenitors, a marker for angiogenesis, was significantly higher in the subchondral bone of ACLT mice relative to sham-operated controls, and this effect was reduced by systemic injection of 1D11 (Fig. 3E). Further demonstration that the articular cartilage was indeed protected by systemic antibody injection was provided by normalization of aggrecan and collagen II expression in chondrocytes, as assessed by immunofluorescence staining in the 1D11-treated ACLT mice relative to sham controls (Fig. 3F–G).

Active TGF- β levels were decreased with injection of 1D11 in ACLT mice

To investigate whether high levels of active TGF- β in the subchondral bone could actually change the levels of circulating TGF- β , we measured the levels of active TGF- β 1 in circulation after ACLT surgery. Indeed, there was notable rise in active TGF- β 1 levels in circulation as early as 7 days post-ACLT in the early stages of OA, reaching peak levels of five times that in the sham group at 2–4 weeks, followed by a slight decrease in the late stages of OA at 8 weeks (Fig. 4A). To examine whether active TGF- β in the circulation can be normalized by 1D11 antibody treatment, 1D11 at a concentration of 5 mg/kg or an equivalent volume of vehicle was injected intravenously three times per week to ACLT or sham-control mice. Similar to the results showing that treatment with the 1D11 effectively prevented uncoupled bone remodeling and protected the cartilage, the abnormal increase in the level of active TGF- β in the circulation seen in ACLT mice was attenuated after treatment with 1D11 injection (Fig. 4B). These results suggested that the level of active TGF- β in the circulation was significantly increased during OA progression, indicating its significant role in the pathogenesis of OA. .

Discussion

In normal healthy joints, the subchondral bone and articular cartilage act as a functional unit, with pressure transmitted from the cartilage to the subchondral bone. Adult bone homeostasis is maintained with coupled remodeling, where TGF- β is activated during osteoclast bone resorption to induce migration of MSCs to the bone remodeling sites for bone formation and angiogenesis.^{31–33} In OA, there is disruption of the temporospatial regulation of osteoblast and osteoclast activity in the subchondral bone, leading to loss of integrity of the subchondral–articular cartilage unit.³⁴ Specifically following an injury like ACLT, increased weight bearing, or muscle weakness from aging, mechanical loading is uneven, resulting in increased osteoclastic activity with subsequent bone resorption, recruitment of MSCs, and resultant osteoblastic activity leading to new bone formation and accompanying angiogenesis. This pathologic process is believed to be mediated by an aberrant increase in TGF- β resulting from increased osteoclastic activity. Disruption of the fine spatiotemporal relationship between osteoclasts and TGF- β results in TGF- β –mediated

recruitment of nestin⁺ mesenchymal stem cells with resultant aberrant bone formation. The role of TGF- β in the uncoupling, recruitment of MSCs, and aberrant bone formation was ably demonstrated by the OA changes seen in CED mice, which overexpress TGF- β .^{8,14} Significant levels of active TGF- β have been found in the synovial fluids of patients with OA.^{35,36} Local administration of TGF- β in knee joints resulted in inflammation and fibrosis, while multiple intra-articular injections of TGF- β result in osteophyte formation.³⁷⁻³⁹

During normal bone remodeling, osteoclasts and their progenitors are found at the sites of bone resorption at the surface. However, as a result of the mechanical loading in OA, there is alteration in the bone microenvironment, resulting in the commitment of osteoprogenitor cells before they reach the surface. Osteoblast differentiation in aberrant locations appears histologically as subchondral bone osteoid islets and alters the thickness of the subchondral plate and calcified cartilage zone, changes known to be associated with osteoarthritis.^{40,41} Several studies have demonstrated that bone marrow MSCs could be identified by nestin and possess all the mesenchymal progenitor potentials, including self-renewal and trilineage differentiation.^{42,43} Further studies have shown that vasculature-associated nestin⁺ bone marrow MSCs are involved in normal bone development and the processes of many bone diseases, including OA.^{44,45} The importance of TGF- β in the recruitment of nestin⁺ MSCs and their interplay in the OA cascade was demonstrated in earlier research as the slowing of OA progression in mouse models in which the TGF- β receptor is knocked out in nestin⁺ MSCs.¹⁴ In our study, we found a decrease in the number of nestin⁺ MSCs, and reduced bone formation and hence “osteoid islets” in the ACLT mouse model after treatment with TGF- β antibody, demonstrating the essential role of TGF- β in pathogenesis and a potential role for TGF- β inhibition in slowing OA progress.

Bone formation is often coupled with angiogenesis, as both nutrients and signaling factors reach their target sites via the circulation.^{46,47} Abnormal vascular congestion in subchondral bone is a known pathologic feature of OA.⁴⁸ The progress of OA is thought to involve osteochondral angiogenesis, with blood vessels breaching the tidemark at the osteochondral junction.⁴⁹ Increased TGF- β signaling in endothelial progenitor cells can promote angiogenesis.⁵⁰ TGF- β may also stimulate the paracrine machinery in MSCs that further facilitates angiogenesis.^{51,52} Our study showed an increased CD31⁺ stain in subchondral bone in the ACLT mouse model, further confirming the role of angiogenesis in OA disease process. This increased angiogenesis was reduced in the ACLT mouse model after treatment with 1D11, a known inhibitor of all three forms of TGF- β . The mechanisms involved in TGF- β regulation of angiogenesis and mobilization of nestin⁺ MSCs needs further investigation.

Articular cartilage is mainly composed of ECM and, to a lesser extent (5%), chondrocytes.⁵³ ECM is made up of collagen fibrils and proteoglycans.⁵⁴ Collagen II is the predominant collagen fibril and aggrecan is the predominant proteoglycan in articular cartilage. Decreases in levels of collagen II and aggrecan are thus associated with degeneration of the articular cartilage and hence pathogenesis of OA.^{55,56} Cartilage is an avascular structure with a nutrient supply provided by the synovial fluid. Despite aberrant increases in TGF- β being implicated in OA pathogenesis, TGF- β has also been shown to be essential in the maintenance of cartilage integrity and repair. While TGF- β was expressed at high levels in

normal cartilage, it was almost absent in OA cartilage, underscoring the essential role of TGF- β in maintaining cartilage integrity.⁵⁷ Injection of TGF- β into naive mice resulted in increased proteoglycan synthesis and hence increased proteoglycan content of the articular cartilage.⁵⁸ Inhibition of endogenous TGF- β results in cartilage degeneration while preventing osteophyte formation. Deletion of TGF- β receptor II in articular chondrocytes leads to a progressive OA-like phenotype in mice.⁵⁹

Thus, it appears that endogenous TGF- β levels are tightly controlled under normal conditions, keeping levels high enough to maintain cartilage integrity but not so high as to cause fibrosis, osteophyte formation, and joint degeneration. Disturbances of this equilibrium by an aberrant increase in TGF- β in the subchondral bone and decreased expression of TGF- β in the articular cartilage after injury play central roles in the pathogenesis of OA. Selective inhibition of the aberrant increase in TGF- β by dose titration, keeping levels as close to physiologic as possible to maintain the functional integrity of the articular cartilage, can potentially slow the progress of OA. We found decreased levels of both collagen II and aggrecan in the articular cartilage in ACLT mice compared to sham controls. This decrease was reduced in ACLT mice treated with 1D11, further confirming the essential role of TGF- β and its antibody in cartilage preservation and, potentially, in OA pathogenesis. The dose of 1D11 selected was shown to have no damaging effects on the cartilage while reducing osteophyte formation and subchondral sclerosis. Higher doses of 1D11 were found to cause cartilage degeneration, potentially through inhibition of endogenous TGF- β .

Targeted inhibition of TGF- β secretion/activation with an optimal dose of antibody could have a role in the pathogenesis of OA. Previous work found that local injection of TGF- β inhibitor resulted in improvement in subchondral bone–articular cartilage homeostasis, slowing the degeneration of articular cartilage and hence OA progression.¹⁴ Systemic administration of 1D11 resulted in reduced TGF- β levels in the circulation, with resultant improvements in subchondral bone architecture in ACLT mice.

We found that 1D11 attenuated OA progression by targeting three subchondral bone pathological features essential to early OA in rodent ACLT models. Specifically, treatment with 1D11 prevented subchondral bone changes, including reduced mobilization of nestin⁺ MSCs, reduced aberrant bone formation through inhibition of TGF- β signaling, and decreased angiogenesis. Most importantly, degeneration of articular cartilage was attenuated. The improvement of the general microenvironment of the subchondral bone and hence the articular cartilage in the ACLT mouse model after treatment with 1D11 provides more evidence of the essential role of TGF- β in the pathogenesis of OA and the potential therapeutic role of inhibiting TGF- β in slowing the progress of OA.

Materials and Methods

Mice

We purchased C57BL/6J (wild-type) mice from Charles River. We transected the ACL in 3-month-old mice to generate a destabilized osteoarthritis animal model as described previously. Briefly, 2-month-old male mice were anesthetized using ketamine and xylazine,

after which we transected the ACL to induce abnormal mechanical loading–associated OA on the left knee. Sham operation was done on independently selected mice by opening the joint capsule on the left knees. For the dosage screening experiments, 3-month-old sham-operated and ACLT mice were assigned to six groups, with 10 mice per group. Three days after surgery, we injected different doses (1, 3, 5, and 10 mg/kg) of TGF- β 1–neutralizing antibody (1D11) or the equivalent volume of vehicle antibody (13C4) (DMSO and PBS) intraperitoneally three times a week, once a week, or once a month for 30 days. Mice were euthanized 15, 30, or 60 days after surgery.⁶⁰

All animals were maintained in the Animal Facility of the Johns Hopkins University School of Medicine. The experimental protocols for both species were reviewed and approved by the Institutional Animal Care and Use Committee of the Johns Hopkins University, Baltimore, Maryland.

Injected antibodies and ELISA

1D11, a murine IgG1 monoclonal antibody, was produced and purified at Genzyme Corporation (Framingham, MA). It neutralizes all three mammalian TGF- β isoforms (β 1, β 2, and β 3). This antibody has a circulatory half-life of 34 h in mice when administered by intraperitoneal injection. An isotype-matched irrelevant murine IgG1 monoclonal antibody, 13C4, also produced by Genzyme Corporation, was used as a control antibody. We determined the concentration of active TGF- β 1 in the conditioned medium using the ELISA Development kit (R&D Systems) according to the manufacturer's instructions.

Histochemistry, immunohistochemistry, and histomorphometry

At the time of euthanasia, we dissected the knee joints and fixed the specimens in 10% buffered formalin for 24 h, decalcified them in 10% ethylenediamine tetraacetic acid (EDTA) (pH 7.0) for 3–4 days, then treated them with 30% glucose for 4 h and embedded them in paraffin. Four-micrometer-thick sagittal-oriented sections of the knee joint medial compartment were processed for H&E and safranin O as well as fast green staining. We used 10 $\times$ modified images to measure the thickness of the calcified cartilage. Calcified cartilage was separated from hyaline cartilage by the tidemark line. We measured the distance from the articular cartilage surface to tidemark as the thickness of the hyaline cartilage and the distance from tidemark to subchondral bone plate (SBP) as the thickness of the calcified cartilage. Immunostaining was performed using a standard protocol. We incubated sections with primary antibodies to mouse nestin (Aves Labs, Inc., 1:300, lot NES0407), osterix (Abcam, 1:600, ab22552), pSmad2/3 (Santa Cruz Biotechnology Inc., 1:50, sc-11769), CD31 (Abcam, 1:100, ab28364), MMP13 (Abcam, 1:40, ab3208), collagen X (Abcam, 1:80, ab58632), collagen II (Abcam, 1:50, ab34712), and aggrecan (Emdmillipore, 1:100, AB1031) overnight at 4 °C. For immunohistochemical staining, we subsequently used a horseradish peroxidase–streptavidin detection system (Dako) to detect the immunoactivity, followed by counterstaining with hematoxylin (Sigma-Aldrich). For immunofluorescent staining, we continued to use secondary antibodies conjugated with fluorescence and incubated the slides, avoiding light, at room temperature for 1 h. We then microphotographed sections to perform histomorphometric measurements on the entire area of the tibia subchondral bone (Olympus DP71). We conducted the quantitative

histomorphometric analysis in a blinded fashion with OsteoMeasureXP Software (OsteoMetrics, Inc.). OARSI scores were calculated as previously described.

Micro-CT analysis

Knee joints were dissected free of soft tissue from mice, fixed overnight in 10% formalin, and analyzed by high-resolution micro-CT (Skyscan1172). The images were reconstructed and analyzed by NRecon v1.6 and CTAn v1.9, respectively. We analyzed parameters of the trabecular bone in the epiphysis using the three-dimensional model visualization software CTVol v2.0. The scanner was set at a voltage of 65 kVp and a resolution of 5.8 μm per pixel. Sagittal images of the tibia subchondral bone were used to perform three-dimensional histomorphometric analyses. The region of interest was defined to cover the whole subchondral bone medial compartment. We used a total of eight consecutive images from the medial tibial plateau for three-dimensional reconstruction and analysis. Three-dimensional structural parameters analyzed included TV (total tissue volume; contains both trabecular and cortical bone), BV/TV (bone volume fraction), SBP (subchondral bone plate), and Tb.Pf (trabecular pattern factor).

Statistics

All statistical analysis were carried out using GraphPad Prism 5 software. Data are presented as the mean $\pm$ SD. We performed comparisons for bone mass, OARSI scores, and microarchitecture among different groups by multifactorial analysis of variance (ANOVA). When ANOVA testing indicated overall significance of the main effects without interactions between them, the differences between individual time points and sites were assessed by post hoc tests. The level of significance was set at $P < 0.05$.

Acknowledgments

All authors meet the International Committee of Medical Journal Editors recommendations. All authors have critically reviewed the manuscript.

This work was supported in part by National Institutes of Health Grants AR 06394, DK 057501 (to Xu Cao), and the 111 Project of MOE. We thank Genzyme Corporation (Framingham, MA) for providing the TGF- β antibody 1D11 and the control antibody 13C4.

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Figure 1.

Systemic neutralization of TGF- β preserves articular cartilage after ACLT. (A–B) Safranin O and fast green staining of sagittal sections of the tibia medial compartment at 60 days after sham operation (A) or ACLT surgery (B) with vehicle antibody: proteoglycan (red), and bone (blue). Scale bars, 200 μ m. (C) Safranin O and fast green staining of tibia articular cartilage and adjacent subchondral bone of knee joints from ACLT mice treated with different doses and frequencies of 1D11 antibody 2 months after ACLT surgery. Scale bars, 200 μ m. (D) OARSI scores at 60 days after sham or ACLT surgery with 1D11 or vehicle antibody injection. (E–F) Immunofluorescent staining and quantitative analysis of the percentage of type X collagen (COL X)⁺ (E) and MMP13⁺ (F) chondrocytes in immunohistochemically stained articular cartilage tissue sections. Scale bars, 100 μ m; $n = 8$ per group. * $P < 0.05$, ** $P < 0.01$ compared to the vehicle-treated sham-operated group. # $P < 0.05$, ## $P < 0.01$ compared to the vehicle-treated ACLT group. Statistical significance was determined by multifactorial ANOVA. All data are reported as the mean $\pm$ SD.

Figure 2.

Systemic neutralization of TGF- β normalizes subchondral bone structure after ACLT. (A–B) Three-dimensional μ CT images of the tibia subchondral bone medial compartment (sagittal view) of mice at 60 days after sham operation (A) or ACLT surgery (B) with vehicle antibody. Scale bar, 1 mm. (C) Three-dimensional μ CT images of the tibia subchondral bone medial compartment (sagittal view) of mice treated with different doses and frequencies of 1D11 antibody 2 months after ACLT surgery. Scale bar, 1 mm. (D–G) Quantitative analysis of CT parameters of subchondral bone 60 days after sham or ACLT surgery with 1D11 or vehicle antibody injection: total tissue volume (TV; D), bone volume fraction (BV/TV; E), thickness of SBPs (SBP Th.; F), and trabecular pattern factor (Tb. Pf; G). $n = 8$ per group. * $P < 0.05$, ** $P < 0.01$ compared to the vehicle-treated sham-operated group. # $P < 0.05$, ## $P < 0.01$ compared to the vehicle-treated ACLT group. Statistical significance was determined by multifactorial ANOVA. All data are reported as the mean $\pm$ SD.

Figure 3. Systemic neutralization of TGF- β normalizes uncoupled bone remodeling and angiogenesis after ACLT. (A–E) Immunohistochemical analysis of pSmad2/3⁺ (A and B), nestin⁺ (C) osterix⁺ (D) and CD31⁺ (E) cells (brown) in mouse tibial subchondral bone or articular cartilage after sham or ACLT surgery with 1D11 or vehicle antibody injection. Arrows indicate positive cells in subchondral bone. Scale bars, 100 μ m (top). On the right is a quantitative analysis of the number of pSmad2/3⁺, nestin⁺, osterix⁺, and CD31⁺ cells per area of bone marrow or articular cartilage (mm²). (F–G) Immunofluorescent analyses (left) and quantification (right) of aggrecan (green; F) and collagen II (green; G) in tibial subchondral bone collected 2 months after sham or ACLT surgery with 1D11 or vehicle antibody injection. Blue in the top row indicates DAPI staining of nuclei. Scale bars, 100 μ m; $n = 8$ per group. * $P < 0.05$ compared to the vehicle-treated sham-operated group; # $P < 0.05$ compared to the vehicle-treated ACLT group. Statistical significance was determined by multifactorial ANOVA. All data are reported as the mean $\pm$ SD.

Figure 4.

Systemic injection of 1D11 neutralized the increased levels of TGF-β in circulation after ACLT. (A) Active TGF-β1 levels in peripheral blood of mice 1 week, 2 weeks, 4 weeks, and 8 weeks after sham or ACLT surgery. $n = 8$ per group. $*P < 0.05$, $**P < 0.01$ compared to the sham-operated group at the corresponding time points. (B) Active TGF-β1 levels in peripheral blood of mice 1 week, 2 weeks, 4 weeks, and 8 weeks after sham or ACLT surgery, following treatment with 1D11 or vehicle antibody injection. $*P < 0.05$, $**P < 0.01$ compared to the vehicle-treated sham-operated group at the corresponding time points. $\#P < 0.05$, $\#\#P < 0.01$ compared to the vehicle-treated ACLT group at the corresponding time points. Statistical significance was determined by multifactorial ANOVA. All data are reported as the mean $\pm$ SD.

Table 1

Assessment for measuring the optimal dosage of 1D11 treatment

Freq.	3 times/week	1 time/week	1 time/month
Conc.			
1 mg/kg	$1 \times 3/W$	$1 \times 1/W$	$1 \times 1/M$
3 mg/kg	$3 \times 3/W$	$3 \times 1/W$	$3 \times 1/M$
5 mg/kg	$5 \times 3/W$	$5 \times 1/W$	$5 \times 1/M$
10 mg/kg	$10 \times 3/W$	$10 \times 1/W$	$10 \times 1/M$

NOTE: $a \times b/W$, a taken b times per week; $a \times b/M$, a taken b times per month; Freq., frequency; Conc., concentration

Table 2

Cartilage thickness changes in different groups and time points

	Hyaline cartilage	Calcified cartilage
Sham-Ve	0.82 ± 0.054	0.31 ± 0.046
ACLT-Ve	0.37 ± 0.138*	0.68 ± 0.017*
1 × 3/W	0.41 ± 0.331	0.65 ± 0.054
1 × 1/W	0.33 ± 0.094	0.67 ± 0.081
1 × 1/M	0.39 ± 0.053	0.65 ± 0.114
3 × 3/W	0.55 ± 0.023 [#]	0.49 ± 0.087 [#]
3 × 1/W	0.42 ± 0.019	0.63 ± 0.131
3 × 1/M	0.39 ± 0.101	0.62 ± 0.061
5 × 3/W	0.81 ± 0.085 ^{##}	0.33 ± 0.094 ^{##}
5 × 1/W	0.64 ± 0.071 [#]	0.47 ± 0.052 [#]
5 × 1/M	0.35 ± 0.079	0.69 ± 0.022
10 × 3/W	0.65 ± 0.124 [#]	0.47 ± 0.037 [#]
10 × 1/W	0.62 ± 0.036 [#]	0.41 ± 0.057 [#]
10 × 1/M	0.35 ± 0.075	0.71 ± 0.061

Note: All data is mean ± SD (mm). The level of significance was set at $P < 0.05$ and indicated by * for the comparison between the vehicle-treated group and the sham group, or # and ## for the comparison between the 1D11-treated group and the 13D4-treated group.