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Regulatory T Cells Protect from Local and Systemic Bone Destruction in Arthritis

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We previously demonstrated the suppressive effects of regulatory T cells (Treg cells) on osteoclast differentiation in vitro. In this article, we show that blood markers of bone resorption inversely correlate with the amount of circulating Treg cells in healthy controls and rheumatoid arthritis patients, further suggesting that Treg cells may control bone destruction in vivo. Indeed, bone marrow from Foxp3-transgenic (Foxp3tg) mice fully protected human TNF transgenic (hTNFtg) mice from TNF-α–induced bone destruction, whereas Foxp3-deficient bone marrow enhanced local and systemic bone loss. The same protective effect was also obtained by treating hTNFtg mice with the CD28 superagonist mAb (CD28 SA), which increased Treg cell numbers. In both models, bone protection by Treg cells was associated with reduced osteoclast numbers, resulting in less bone-resorbing activity. Reduced osteoclast numbers were not caused by an intrinsic defect in osteoclast differentiation because osteoclast precursors from hTNFtg/Foxp3tg chimeras responded normally to M-CSF and receptor activator of NF-κB ligand. Although a decrease in the clinical signs of arthritis was observed in Foxp3tg bone marrow–transferred and CD28 SA–treated hTNFtg mice, the bone-protective effect of Treg cells was independent of the suppression of inflammation, as demonstrated by the increased systemic bone density observed in wild-type mice treated with CD28 SA. This work demonstrated that increasing Treg cell numbers improved clinical signs of arthritis and suppressed local and systemic bone destruction. Thus, enhancing the activity of Treg cells would be beneficial for the treatment of inflammation–induced bone loss observed in rheumatoid arthritis. The Journal of Immunology, 2010, 184: 000–000.

Rheumatoid arthritis (RA) is a common autoimmune disease affecting 1% of the population. RA is characterized by a chronic inflammation of the synovium (synovitis) that spreads to cartilage and bone, leading to the destruction of the surface cartilage and subchondral bone (1, 2). In addition to the local bone and cartilage destruction at the site of inflammation, the second hallmark of RA is the development of systemic osteopenia associated with an enhanced risk for osteoporotic fractures. Local and systemic bone loss in arthritis is based on a profound imbalance between bone formation and bone resorption. Pivotal cytokines in RA, such as TNF-α, promote this deleterious imbalance in bone metabolism and contribute to enhanced bone destruction (3). One of the key cytokines is TNF-α, because its blockade has emerged as a highly effective therapy in RA, and overexpressing human TNF in mice leads to severe arthritis very similar to the human pathology (4).

Although the reason for the development of the early autoimmune reaction is unknown, the mechanisms that connect immune activation to bone destruction are better defined and built the basis of a new field of research called osteoimmunology (1). In addition to forming the skeleton, bone is the main hematopoietic organ homing the hematopoietic stem cells (3, 5). In agreement, the recruitment of hematopoietic stem cells and, therefore, the early step of differentiation of lymphoid cells depend on bone remodeling that occurs throughout life in vertebrates. Conversely, modulation of bone remodeling by immune cells was suggested by Horton et al. (6) in 1972, showing the capacity of immune cells to influence osteoclast differentiation and activity. Nearly 30 y later, receptor activator of NF-κB ligand (RANKL) was identified as the essential factor for osteoclast differentiation. RANKL, a member of the TNF family, is expressed on the mesenchymal cells and on activated T cells, which illustrates the tight link between immune activation and bone (7).

Several studies investigated the role of T cells in bone homeostasis, especially osteoclast differentiation. It was first shown that activated CD3+ T cells that express RANKL were capable of supporting osteoclast differentiation in vitro (8). In line with this, the surface expression of RANKL by activated CD4+ T cells was also shown to stimulate osteoclast differentiation in vitro (9). The activating effect of T cells on osteoclast differentiation could additionally be boosted by cytokines like TNF-α
and IL-1 and -18, which upregulate the surface expression of RANKL on T cells, thereby driving osteoclast differentiation (10). However, IFN-γ that is coexpressed with RANKL by Th1 cells was shown to block RANKL signaling by promoting TNFR-associated factor 6 degradation; therefore, it inhibited osteoclast differentiation (11). The suppressive effect of IFN-γ on osteoclast differentiation questioned the in vivo relevance of the in vitro pro-osteoclastogenic activity of activated T cells. Indeed, it was recently shown that purified Th1 and Th2 cells inhibited osteoclast differentiation when cocultured with monocytes in the presence of M-CSF and RANKL (12). This inhibitory effect was based on the cytokines produced by these Th cell subsets, such as IFN-γ and IL-4. However, the same study identified Th17 cells as the key T cell subset that enhanced osteoclast differentiation. IL-17 produced by Th17 cells indirectly enhanced osteoclast differentiation by upregulating RANKL expression on osteoblasts and bone marrow stromal cells (12). Indeed, Th17 cells, in conjunction with other cell types producing IL-17, may trigger bone and cartilage loss in disease, such as RA; this is supported by 1) the presence of cells expressing IL-17 in the synovium of RA patients, 2) the ability of IL-17 to mediate cartilage destruction (13), and 3) the stimulating effect of IL-17 derived from the synovial fluid of RA patients on osteoclast differentiation (14, 15).

Following the concept that T cell activation contributed to bone loss in arthritis, one can hypothesize that regulation of T cell activation could stop bone loss in arthritis. Indeed, a potential role for naturally occurring regulatory T cells (Treg cells) in RA has been proposed. Treg cells are essential modulators of self-tolerance, as shown by the fatal lymphoproliferative syndrome that develops in humans and mice carrying an inactivating mutation of Foxp3, an essential transcription factor of Treg cells (16, 17). Surprisingly, large numbers of Treg cells with suppressive activity in vitro are found in the inflamed synovium of RA patients. However, their ability to suppress cytokine production by effector T cells seems to be impaired. This impaired suppressive function was overcome by blocking TNF-α (18). In agreement, TNF-α was shown to downregulate the function of human Treg cells via inhibition of Foxp3 expression (19). These data suggested the imbalance between proinflammatory Th17 cells and Treg cells as being determinant in RA (20, 21). Also, although Treg cell depletion was shown to aggravate the symptoms of arthritis, amelioration of disease was reported in several T cell-driven animal models of arthritis following the adoptive transfer of Treg cells, suggesting a therapeutic potential of restoring Treg cells activity in arthritis.

However, demonstration of the beneficial effects of Treg cells on arthritis have been confined to T cell-dependent models of immune-mediated disease, which allowed the greatest chance to detect a strong immunoregulatory effect leading to inhibition of immune activation, inflammation, and, as a consequence, bone damage (22, 23). However, these immune-mediated disease models did not allow the separation of potential direct effects of Treg cells on bone, which is suggested by their ability to directly inhibit osteoclast differentiation in vitro (24). Therefore, we took

![Figure 1](http://www.jimmunol.org/)

**Figure 1.** FOXP3+ T cells in peripheral blood of normal human subjects (n = 35) and RA patients (n = 35) are inversely related to serum markers of osteoclastogenesis. TRAP5b ELISA analysis correlated with flow cytometry analysis of CD4+CD25+FOXP3+ T cells in healthy controls (Spearman r = −0.7311; p < 0.0001) and RA patients (r = −0.748; p < 0.0001) (A), CTX-I (Spearman r = −0.65; p < 0.0001) and RA patients (Spearman r = −0.64; p < 0.0001) (B), and osteocalcin levels of healthy controls (Spearman r = −0.2955) and RA patients (Spearman r = −0.19) (C).
advantage of the human TNF transgenic (hTNFtg) mouse model, which mimics human RA by the overexpression of TNF and results in a non-T cell–dependent inflammatory arthritis. We first reconstituted irradiated hTNFtg mice with bone marrow from Foxp3-transgenic (Foxp3tg) mice, which display increased Treg cell numbers, and from scurfy mutant mice, which lack Treg cells, and analyzed the development of clinical signs of arthritis as well as local and systemic bone destruction. In addition, we used a pharmacological approach by treating hTNFtg mice with CD28 supernagonist (CD28 SA), which was shown to increase Treg cell numbers in vivo. We found that Treg cells blocked inflammation and completely inhibited bone erosion in TNF-mediated arthritis as well as systemic bone loss. Moreover, the induction of Treg cells by targeting CD28 increases bone mass in arthritic mice and in nonarthritic wild-type (WT) mice, suggesting that Treg cells have a direct effect in controlling bone mass, which goes beyond their immunoregulatory role.

Materials and Methods

**Mice**

Foxp3tg mice (strain 2826, C57BL/6), the scurfy mutant (sf/Y), and hTNFtg mice (strain Tg197) were described previously (4, 17, 25). All animals were performed in a specific pathogen-free facility. All animal experiments were performed with the approval of the ethic local authorities.

**FACS analysis of surface molecules**

For extracellular staining, 1 × 10⁶ cells/staining were washed with 1 ml FACS buffer, resuspended in 100 μl FACS buffer, and incubated with saturating amounts of PE-, FITC-, PerCP-, PCy7-, or allophycocyanin-labeled Abs (BD Biosciences, Heidelberg, Germany) for 30 min at 4˚C in the dark. One aliquot of the cells was parallel stained with corresponding control isotype Ab to monitor for the specificity of the staining. After, cells were washed twice with 1 ml FACS buffer, resuspended in 300 μl FACS buffer, and analyzed. Bone marrow chimera animals were analyzed with a FACSCalibur, and mice treated with CD28 SA Ab were analyzed with a FACSCanto Flow Cytometer (both from BD Biosciences). For intracellular staining 1 × 10⁶ cells were fixed and permeabilized according to the manufacturer’s instructions (eBioscience, Frankfurt, Germany).

**Isolation and culture of osteoclast precursors**

Bone marrow was isolated from 5–8-wk-old C57BL/6 WT, Foxp3tg, or sf/Y (scuffy) mice by flushing femoral bones with complete media. Alternatively, osteoclast precursors were isolated from bone marrow–derived cell suspensions using CD11b microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer’s instructions. The purity of isolated monocytes was assessed by flow-cytometry analyses using CD11b-FITC–labeled Abs (Miltenyi Biotec). CD11b+ monocytes were plated in 96-well plates (2.5 × 10⁴/well) or 48-well plates (5 × 10⁴/well) in αMEM supplemented with 10% FCS, 30 ng/ml M-CSF, and 50 ng/ml RANKL (R&D Systems, Wiesbaden-Nordenstadt, Germany). Medium was changed after 72 h. Osteoclast differentiation was evaluated by staining fixed cells for tartrate-resistant acid phosphatase (TRAP) using a Leukocyte Acid Phosphatase Kit (Sigma-Aldrich, Taufkirchen, Germany), according to the manufacturer’s instructions.

**Bone-resorption experiments**

Calcified matrix-resorption activity of the osteoclasts was tested using BioCoat Osteologic plates (BD Biosciences). von Kossa staining was performed with the BioCoat Osteologic Discs to visualize the resorption pits. Resorption pit area was assessed using the Nikon NIS-Elements 3.0 Software (Nikon, Düsseldorf, Germany).

**RT-PCR**

Isolation of RNA was done with the standardized combination of TRizol reagent. RNA concentration was measured before genomic DNA was digested with DNase I (Fermentas, St. Leon-Rot, Germany), and cDNA was synthesized in the presence of RNase inhibitor. For SYBR Green-based detection, a dissociation curve was carried out by one cycle following the last amplification cycle to control for the specificity of PCR amplification: 95˚C for 15 s, 60˚C for 30 s, and 95˚C for 15 s. Relative quantification was performed by calculating the difference in cross-threshold values (ΔCt) of the gene of interest and a housekeeping gene according to the equation 2⁻^ΔCt. In some experiments, the relative expression values were normalized to the expression values in the control condition.

**ELISA**

Serum levels of murine osteocalcin and murine C-terminal telopeptide α1-chain of type I collagen (Ratlaps, CTX-I, Nordic Bioscience, Herlev, Denmark), human TRAP5b (Quidel, San Diego, CA), osteocalcin (Quidel), and C-terminal telopeptide α1-chain of type I collagen (CrossLaps, CTX-I, Nordic Bioscience) were analyzed by ELISA. All ELISA analyses were performed according to the manufacturer’s instructions.

**Micro-computerized tomography imaging**

Micro-computerized tomography (µCT) images of tibias were acquired on a laboratory cone-beam µCT scanner developed at the Institute of Medical Physics for ultra high-resolution imaging (26). It uses a μ-Focus X-ray tube (Hamamatsu, Herrsching, Germany) and a two-dimensional cooled charge-coupled device detector array (1024 × 1024 elements, 19-μm pitch; Roper Scientific, Tucson, AZ) with a dynamic range of 16 bit. A fiber optics taper enlarges the sensitive input area of the charge-coupled device by a factor of three. The detector and sample stage can be linearly translated independently with respect to each other and with respect to the x-ray source, providing variable magnification of the object. For the current study, the following acquisition parameters were used: voltage: 40 kV, x-ray current: 250 μA, exposure time: 5000 ms/projection, 720 projections, matrix: 1024 × 1024, voxel size in reconstructed image: 9 μm. Images were analyzed using a plug-in programmed for Amira 4.1.2. (Visage Imaging, San Diego, CA) with the following histomorphometric parameters: trabecular volume/total volume (BV/TV), trabecular thickness, and trabecular number.

**Bone histomorphometry**

Histomorphometry was performed on methacrylate-embedded undecalciﬁed plastic sections following von Kossa and Goldner staining. Quantifications

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**FIGURE 2.** Treg cells modulate inflammation in TNF-mediated arthritis. *A.* Analysis of CD25⁵Foxp³ cells in the bone marrow and spleen of the transplanted mice. *B.* Clinical markers from irradiated hTNFtg mice reconstituted with bone marrow from WT, sf/Y, or Foxp3tg mice. Paw swelling, grip strength, and body weight are shown. sf/Y, scurfy.
were performed by digital image analysis (OsteoMeasure; OsteoMetrics, Decatur, GA). Histological analyses were performed on formalin-fixed, decalcified, paraffin-embedded tissue sections stained with H&E, TRAP, or toluidine blue. Synovial inflammation, osteoclast numbers, and cartilage destruction were quantified by digital image analysis (OsteoMeasure).

**Dynamic labeling of bone**

At 11 wk of age, all mice were given two injections of calcein green (Sigma-Aldrich) at a dose of 30 mg/kg body weight 7 d apart and were killed 2 d after the final injection. Left tibial bones were embedded in methoxymethylmetacrylate. Measurements were performed on the entire marrow region within the cortical shell using an image-analysis system (OsteoMeasure), and the mineral apposition rate (MAR) (μm/day) was calculated.

**Clinical assessment**

Arthritis (paw swelling and grip strength) was assessed using a semi-quantitative score, as described previously (27).

**Immunohistology**

Deparaffinized ethanol-dehydrated tissue sections were pretreated with high-temperature unmasking solution (20 min in citrate buffer [pH 6]) for staining with a mAb against Foxp3 (clone FJK-16s; eBioscience).

**Bone marrow transplantation**

Recipient hTNFtg mice (6 wk old) were irradiated (11 Gy) using orthovoltage irradiation (Stabilipan, Siemens, Erlangen, Germany) at 250 kV/15 mA 40 cm focus – surface distance at a dose rate of 1.15 Gy/min. For irradiation, the mice were subjected to inhalation anesthesia (Forene, Abbott, Wiesbaden-Delkenheim, Germany), which was performed during the irradiation process in a closed fixture made of Plexiglas. This fixture was mounted on a Plexiglas block (thickness = 50 mm) to achieve full-reflection scattering. The next day, mice were reconstituted by i.v. injection of 5 × 10⁶ bone marrow cells in Medium 199 (Sigma-Aldrich) containing 5 ml 1 M HEPEs buffer (Life Technologies, Carlsbad, CA), 5 ml (1 mg/ml) DNAse (Sigma-Aldrich), and 40 μl (50 mg/ml) gentamicin (Sigma-Aldrich); they were analyzed 6 wk after transplantation.

**Blood donors**

Serum levels of CTX-I, TRAP5b, and osteocalcin were analyzed in 35 healthy subjects (mean age, 55.8 ± 8.1 y; 57% females) and 35 patients with RA (mean age, 57.9 ± 6.4 y; 63% females). Treg cells were analyzed with a Treg detection kit (Miltenyi Biotec) using Abs against CD4, CD25, and FOXP3. Cells were gated for CD4⁺ cells, and Treg cells were identified as CD25highFOXP3⁺ cells. Studies were approved by the local ethical committee of the University of Erlangen, and written informed consent was given by all blood donors.

**CD28 SA application**

At weeks 6 and 9, hTNFtg and WT mice received an intraperitoneal injection of 200 μg CD28 SA (28, 29). Mice were observed for 6 wk.

**Adoptive Treg cell transfer**

For the adoptive Treg cell transfer model in hTNFtg mice, sex-matched littermates were injected once i.v. at 9 wk of age (3 wk after the onset of inflammatory arthritis) with 1 × 10⁶ isolated WT CD4⁺CD25⁺ T cells or with PBS as control. Isolation of Treg cells and analysis of the purity and functionality of the cells were performed, as described previously (24). Spleens were isolated from C57BL/6 WT mice and homogenized through a 70-μm stainless steel mesh to obtain a single-cell suspension. Erythrocytes

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**FIGURE 3.** Treg cells protect from local bone erosion in TNF-mediated arthritis. A, Histological sections of the tarsal joints of hTNFtg mice after bone marrow transfer from WT, sf/Y, or Foxp3tg mice stained with H&E (upper row, original magnification ×2), TRAP (middle row, original magnification ×2), and toluidine blue (lower row, original magnification ×20). Scale bar, 500 μm. B, Histomorphometric assessment of synovial inflammation, cartilage destruction, and osteoclast numbers. C, Immunohistochemical quantification of the frequency of FoxP3⁺ cells in the joints of transplanted mice (n = 10). Representative foxp3 staining for each group of mice is shown (original magnification ×40). *p < 0.05; **p < 0.01; ***p < 0.001. sf/Y, scurfy.
were lysed with NH4Cl. CD25+ and CD25\(^+\) CD4+ T cell populations were isolated from the splenic cell suspensions using a microbead-based Regulatory T Cell Isolation Kit (Miltenyi Biotec), according to the manufacturer’s instructions.

**Statistical analysis**

All statistical analyses were performed, using the Student t test or one- or two-way ANOVA followed by the Tukey test, with GraphPad Prism 4.0 Software (GraphPad, San Diego, CA). Data are represented as mean ± SEM, unless otherwise stated. All experiments were done with 10 mice per group, unless otherwise stated.

**Results**

**Foxp3\(^+\) T cells in peripheral blood of normal human subjects and RA patients are inversely related to serum markers of osteoclastogenesis**

Based on our previous in vitro data, we hypothesized that Treg cells may have a beneficial effect on bone in vivo. We found evidence for such a concept when investigating cohorts of healthy individuals and RA patients and analyzing markers of bone metabolism as well as peripheral Treg cell counts. Interestingly, the number of CD4\(^+\)CD25\(^{\text{high}}\)FOXP3\(^+\) T cells in peripheral blood was inversely related to serum markers of osteoclastogenesis, such as TRAP5b (Spearman \(r = -0.7311, p < 0.0001\)), and bone resorption, such as CTX-I (Spearman \(r = -0.65, p < 0.0001\)), in healthy humans and in RA patients (Fig. 1A, 1B). In contrast, no inverse correlation with markers of bone formation, such as osteocalcin, was observed (Fig. 1C). Thus, decreased circulating Treg cell numbers are associated with increased osteoclast formation and bone resorption in humans. This observation suggested that Treg cells might exert a protective effect against bone resorption in vivo, which could emerge as a novel concept to protect from inflammation-induced bone loss.

**Treg cells inhibit TNF-\(\alpha\)–induced inflammatory arthritis**

TNF-\(\alpha\) plays an essential role in chronic inflammatory diseases and links inflammation to degradation of bone by stimulating osteoclastogenesis. Overexpression of human TNF-\(\alpha\) in mice (hTNFtg) leads to destructive arthritis and severe systemic osteopenia (4, 30). To address the role of Treg cells in inflammatory bone loss, we reconstituted hTNFtg mice with bone marrow.
from WT, Foxp3-deficient scurfy (sf/Y), or Foxp3tg mice. The efficiency of the reconstitution was addressed by analyzing the proportion of CD25⁺Foxp3⁺ T cells in the spleen and bone marrow of the recipient mice. Compared with reconstitution with bone marrow from WT donors, a significant decrease in CD25⁺Foxp3⁺ T cells was observed in the spleen and the bone marrow of hTNFtg mice receiving bone marrow from sf/Y donors, and a significant increase in CD25⁺Foxp3⁺ T cells was observed when donor cells came from Foxp3tg mice (Fig. 2A). Having established the efficiency of the bone marrow transfer, we analyzed its effect on the development of TNF-induced arthritis. Progressive paw swelling, loss of grip strength, and loss of body weight are clinical symptoms that characterize the development of inflammatory arthritis in hTNFtg mice. All of these signs were also found in hTNFtg/WT chimeras (Fig. 2B). For all three parameters, the severity was more pronounced in hTNFtg/sf/Y chimeras, but it significantly improved in hTNFtg/Foxp3tg chimeras (Fig. 2B). Therefore, although decreasing Treg cell numbers aggravated the clinical signs of arthritis, increasing Treg cell numbers were beneficial.

**Treg cells protect from TNF-α–induced systemic bone loss**

We next performed histological analysis of the tarsal joints of chimeric mice to determine whether Treg cells modulate local bone destruction and cartilage breakdown. An almost complete preservation of joint architectures was observed in hTNFtg/Foxp3tg chimeras, whereas hTNFtg/sf/Y chimeras exhibited the most severe structural damage, with dramatic bone loss as well as cartilage destruction (Fig. 3A, 3B). Moreover, osteoclast numbers in the joint were increased in hTNFtg/sf/Y chimeras and drastically reduced in hTNFtg/Foxp3tg chimeras (Fig. 3A, 3B), which inversely correlated with the number of Foxp3⁺ Treg cells in the joints (Fig. 3C).

These data indicated that Treg cells are protective against inflammatory arthritis and exerted a local antosteoclastogenic effect at those sites, where osteoclasts are formed.

**Treg cells protect from TNF-α–induced systemic bone loss**

The second hallmark of inflammatory arthritis is the development of a generalized osteopenia due to increased general bone destruction by osteoclasts and decreased bone formation by osteoblasts. Therefore, we analyzed systemic bone architecture of hTNFtg mice following adoptive transfer with bone marrow from WT, sf/Y, or Foxp3tg mice. Micro-computed tomography μCT imaging clearly indicated that reconstitution with bone marrow from sf/Y mice significantly aggravated the osteopenic phenotype of hTNFtg mice (Fig. 4A, 4B). In contrast, hTNFtg/Foxp3tg chimeras maintained a greater bone mass and were resistant to systemic bone loss (Fig. 4A, 4B). Structural analysis showed that, while trabecular thickness was unaffected, regulation of bone mass was caused by significantly higher trabecular numbers in hTNFtg/Foxp3tg and lower trabecular numbers in hTNFtg/sf/Y chimeric mice (Fig. 4B). Histomorphometry was performed to determine the cellular mechanisms responsible for Treg cell-induced changes in bone mass in the chimeric mice. TRAP staining revealed decreased numbers of osteoclasts in hTNFtg/Foxp3tg chimeras and increased numbers in hTNFtg/sf/Y chimeric mice; this was confirmed by quantifying osteoclast parameters, such as osteoclast numbers and osteoclast-covered bone surface (Fig. 4C). No change in osteoblast numbers or osteoblast-covered bone surface could be detected by histology (Fig. 4D). However, increased MAR was observed in hTNFtg/Foxp3tg chimeric mice (Fig. 4E, 4F). These data indicated that Treg cell activity is a key player in skeletal homeostasis during inflammation and that increasing Treg cell numbers protected against systemic bone loss. **FIGURE 6.** CD28 SA protects systemic bone mass in hTNFtg mice. Flow cytometry analysis of CD4⁺CD25⁺Foxp3⁺ cells in spleen (A) and bone marrow (B) of CD28 SA- and isotype control-treated mice. C. Clinical parameters (paw swelling, grip strength, and body weight measurements) from hTNFtg mice treated with CD28 SA twice within 6 wk. D. Histomorphometric assessment of synovial inflammation, cartilage destruction, and osteoclast numbers in the tarsal joints. E. Structural parameters of trabecular bone (BV/TV, trabecular number, and trabecular thickness) from tibia of CD28 SA-treated hTNFtg mice at week 12. F. Quantitative histomorphometry of Oc.N/mm and Oc.S./BS. G. Serum levels of CTX-I as marker for bone resorption and osteocalcin as marker for bone formation. H. Quantitative histomorphometry of Ob.N/mm and Ob.S./BS. Ob.N/mm: osteoblast numbers normalized to trabecular bone perimeter; Ob.S./BS, osteoblast surface normalized to bone surface; Oc.N/mm; osteoclast numbers normalized to trabecular bone perimeter; Oc.S./BS; osteoclast surface normalized to bone surface.
destruction by inhibiting osteoclast differentiation and restoring the bone-forming activity of osteoblasts. To assess the potential of Treg cells to protect against bone destruction in a therapeutic setting, hTNFtg mice were injected with 1 × 10^6 isolated Treg cells 3 wk after the onset of inflammatory arthritis. Histomorphometrical analysis of the tibia 3 wk after the Treg cell transfer showed an increase in systemic bone density (Fig. 4G) that was associated with decreased osteoclast parameters, such as osteoclast numbers and osteoclast-covered bone surface (Fig. 4H). The success of the Treg cell transfer into hTNFtg mice was confirmed by FACS analysis, which showed an increased proportion of CD4^+CD25^+Foxp3^+ cells in the spleen (Fig. 4I).

**Treg cell numbers regulate the pool of osteoclast precursors but do not change their intrinsic differentiation capacity**

Our data indicated that Treg cells protect against TNF-α–induced local and systemic bone destruction by decreasing the number of differentiated osteoclasts. Thus, we analyzed whether the variation in osteoclast numbers observed in the chimeric mice was caused by an alteration in the differentiating properties of osteoclast precursors. Osteoclast differentiation was first performed with total bone marrow cultured in the presence of M-CSF and RANKL, revealing increased osteoclast formation in hTNFtg/sf/Y chimeras (Fig. 5A). This effect was most likely based on altered redistribution of CD11b^+ osteoclast precursors with greater numbers of CD11b^+ cells in the bone marrow and spleen of these mice (Fig. 5B). Indeed, direct in vitro osteoclast assays with CD11b^+ purified cells from hTNFtg/Foxp3tg, hTNFtg/sf/Y, and hTNFtg/wt chimeras showed no difference in osteoclast differentiation or in the resorptive activity, confirming that Treg cells did not affect the intrinsic osteoclastogenic potential of mononuclear cells (Fig. 5C, 5D).

**CD28 SA ameliorates TNF-induced arthritis and systemic bone loss**

Our data suggested that increasing numbers of activated Treg cells could be a therapeutical tool to mitigate TNF-mediated arthritis and bone damage in the context of arthritis. To test this hypothesis, we treated hTNFtg mice with CD28 SA Ab to selectively target and expand Treg cells in mice (28). Mice were injected twice during the study period, and the increase in the frequency of CD4^+CD25^+Foxp3^+ cells in the spleen and bone marrow in the CD28 SA-treated mice was documented by FACS analysis (Fig. 6A, 6B). When progressive clinical signs of arthritis were analyzed, paw swelling, grip strength, and loss of body weight were all ameliorated in hTNFtg mice treated with CD28 SA compared with untreated controls (Fig. 6C). Histological analysis of the tarsal joints showed significant protection of joint architectures in CD28 SA-treated hTNFtg mice. They exhibited less bone erosion, less cartilage destruction, and a reduced number of osteoclasts (Fig. 6D). In addition, systemic bone density was significantly increased in CD28 SA-treated hTNFtg mice, as indicated by the increased BV/TV, which was mainly based on an increase in trabecular numbers in treated mice (Fig. 6E). The preservation of local joint architecture and systemic bone mass suggest a bone-protective role of Treg cells in CD28 SA-treated mice. At the cellular level, we observed decreased osteoclast numbers and osteoclast-covered bone surface in CD28 SA-treated hTNFtg mice (Fig. 6F), indicating impaired bone resorption that was confirmed by reduced CTX-I in the serum. In contrast, osteocalcin serum levels, a marker for bone formation, remained unchanged (Fig. 6G). Moreover, no change in osteoblast numbers or osteoblast surface per bone surface was detected (Fig. 6H).

**Increase in bone mass in CD28 SA-treated WT mice**

To determine whether the protective effect of increasing numbers of Treg cells on bone destruction was purely linked to a decrease in inflammation, we treated WT mice with CD28 SA. We first confirmed the increase in CD4^+CD25^+Foxp3^+ Treg cells in the treated mice by FACS analysis (Fig. 7A). μCT measurements of tibias from WT controls and sex- and age-matched littermates treated with CD28 SA revealed a significant increase in bone mass (BV/TV) in treated mice (Fig. 7B, 7C). The phenotype was again based on greater numbers of bony trabeculae (Fig. 7B, 7C). Decreased osteoclast numbers and osteoclast-covered bone surface indicated an impaired bone resorption in CD28 SA-treated mice (Fig. 7D) but an unchanged pool of osteoclast precursor cells (Fig. 7E). Moreover, decreased serum levels of CTX-I revealed impaired bone resorption, in contrast to unchanged osteocalcin serum levels (data not shown) used as markers for bone formation in these mice (Fig. 7F).

**Discussion**

In this article, we showed that Treg cell numbers could inhibit inflammation in a standard TNF-dependent T cell-independent model of chronic arthritis and block structural damage, which results from the detrimental effects of TNF on bone and cartilage. We showed that increasing Treg cell numbers by bone marrow transfer from Foxp3tg mice in a preventive setting and adoptive Treg cell transfer as a therapeutic approach after the onset of

**FIGURE 7.** CD28 SA increases bone mass in WT mice. A. Increased frequency of CD4^+CD25^+Foxp3^+ cells in the bone marrow and spleen of CD28 SA-treated WT mice. B. μCT imaging of the tibia of isotype control- and CD28 SA-treated mice. C. Structural parameters of trabecular bone (BV/TV, trabecular number, and trabecular thickness) from tibia of CD28 SA-treated mice at week 12. D. Quantitative histomorphometry of Oc.N/mm and Oc.S./BS. E. Flow cytometry analysis of osteoclast progenitors (CD11b^+ cells) in the bone marrow (n = 5). F. Serum levels of CTX-I as a marker of bone destruction. Oc.N/mm; osteoclast numbers normalized to trabecular bone perimeter; Oc.S./BS; osteoclast surface normalized to bone surface.
arthritis inhibited or ameliorated local and systemic bone destruction during arthritis. Moreover, increased Treg cell activity fostered bone, even in the absence of inflammation, suggesting that part of the bone-protective effects of Treg cells is indeed mediated by direct interference with osteoclasts and osteoclast-mediated bone resorption and is not secondarily due to improvement of inflammatory signs of arthritis.

The role of Treg cells in the development of RA is still a matter of debate. Although the frequency of Treg cells in the synovial fluid of RA patients is enhanced compared with their frequency in the peripheral blood and the blood of healthy donors (31), recent studies suggested that the function of Treg cells may be impaired in RA patients. They were still able to suppress the proliferation of CD4+CD25+ T cells (32) but without suppressing the secretion of proinflammatory cytokines, such as IFN-γ (18). Interestingly, anti–TNF-α therapy in RA patients fully restored the suppressive capacity of Treg cells on both the proliferation of CD4+CD25+ T cells and cytokine secretion (18). Mechanistically, anti–TNF-α therapy did not restore the suppressive phenotype of defective Treg cells but rather give rise to a new TGF-β-induced subpopulation of Treg cells (CD4+CD25+CD62L−) (33). In addition, TNF-α was shown to upregulate the expression of TNFR type II on Treg cells, and binding of TNF-α to TNFR type II reversed their suppressive activity by downregulating FOXP3 (19). These data suggested that TNF-α created an environment that disfavors Treg cell function. Our new data showed that without directly blocking TNF-α, enhancement of Treg cell numbers was sufficient to protect mice against TNF-α-induced arthritis. Indeed, endogenous Treg cells could be efficiently recruited in CD28 SA-treated hTNFtg mice. It was demonstrated that CD28 SA was capable of expanding functional CD4+CD25+ Treg cells (34), which were effective in the treatment of experimental autoimmune encephalomyelitis (35). Thus, our data reinforced previous observations that adoptive transfer of CD4+CD25+ Treg cells could suppress the development of arthritis in the mouse model of collagen-induced arthritis (23). Indeed, the adoptive transfer of WT Treg cells after the onset of arthritis in hTNFtg mice was able to ameliorate the systemic bone loss by decreasing osteoclast numbers. Conversely, and in agreement with the increased signs of arthritis that we observed following the transfer of bone marrow isolated from the scurvy mice, depletion of CD4+CD25+ Treg cells accelerated the onset of collagen-induced arthritis in mice (36) as well as in a K/BxN mice expressing both the T cell receptor transgene KRN and the MHC class II molecule Aβ7 model (37). However, in both of these immune-mediated disease models, the development of arthritis is primarily driven by the activity of the immune cells that are suppressed by Treg cells. The originality of the hTNFtg mice resides in the fact that inflammation is not primarily caused by the hematopoietic cells, as shown by the failure of bone marrow isolated from hTNFtg mice to transfer the disease to WT recipients (38).

Previous studies did not address the possibility that Treg cells, in addition to suppressing T cell responses, directly regulate bone homeostasis in vivo. In our present study, we observed a protective effect of Treg cells on TNF-induced local bone erosion and systemic bone loss in a T cell-independent arthritis model. Protection from inflammation-induced local bone erosion by Treg cells was reported in the T cell-dependent collagen-induced model of arthritis (39). Treg cells also mitigated inflammation in hTNFtg mice, which could contribute, in part, to the protective effect on local bone destruction. In addition, regulation of bone mass by Treg cells extends to systemic bone mass, which is increased with high numbers of Treg cells or decreased in the absence of Treg cells. Moreover, very similar effects were found in WT mice by using a pharmacological approach with CD28 SA. Bone mass increased with increased numbers of Treg cells, and this effect was observed in the absence of an inflammatory process, suggesting that Treg cells exerted direct effects on bone metabolism. The increase in systemic bone mass observed in WT and hTNFtg mice treated with CD28 SA was consistent with the observed decrease in serum levels of markers for bone destruction and unchanged serum markers of bone-formation markers. This hypothesis is reinforced by recent studies showing that Treg cells can directly block osteoclast differentiation in vitro (24, 39, 40). Interestingly, it was demonstrated that the defects in Treg cell function in RA patients resulted from defects in CTLA-4 surface expression (41). These data substantiated our former in vitro results, showing that the suppressive mechanism of Treg cells on osteoclast differentiation depends on cell–cell contact mediated by CTLA-4 (24, 42). The potential clinical relevance of our data is underscored by the negative correlation between the number of peripheral Treg cells and the level of bone-resorption markers in normal human subjects, as well as in patients with RA. We are aware of the TGN1412 (Tegenero Immuno Therapeutics) study with CD28 SA in humans (43), in which a cytokine storm, rather than an anti-inflammatory response, was observed. However, Beyerheld et al. (35) clearly showed that in rodents, CD28 SA preferentially expanded Treg cells, as shown by the direct monitoring of Treg cell proliferation upon CD28 SA stimulation in vivo. In addition, the study by Gogishvili et al. (29) showed that CD28 SA treatment increased the number of Foxp3+ T cells in the spleen, as well as their suppressive capacity. Therefore, in mice, it seemed that the effective stimulation of Treg cells by CD28 SA treatment was able to prevent the cytokine storm that was observed in humans. This is highlighted in our experiments in which neither the CD28 SA-treated hTNFtg mice nor the WT mice showed any visible adverse reactions. These studies clearly show that in mice, in contrast to the situation in humans, CD28 SA can be successfully used to expand and activate Treg cells.

In summary, we showed that Treg cells protect against local and systemic TNF-mediated bone loss and that the protective effects of Treg cells on bone extend to noninflammatory conditions. This protective effect on bone is due to inhibition of osteoclast differentiation and can also be obtained by pharmacological expansion of the Treg cell population, despite the presence of TNF-α. Therefore, expansion of Treg cells would be beneficial for the treatment of inflammation-induced bone destruction as observed in patients with RA, as well as for systemic bone loss in the context of inflammatory and noninflammatory conditions.

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References


