Regulatory T cells in the bone marrow microenvironment in patients with prostate cancer

Ende Zhao, Lin Wang, Jinlu Dai, Ilona Kryczek, Shuang Wei, Linda Vatan, Saleh Altuwaijri, Tim Sparwasser, Guobin Wang, Evan T. Keller and Weiping Zou

Department of Surgery; University of Michigan; Ann Arbor, MI USA; Department of Surgery; Central Laboratory; Union Hospital; Tongji Medical College; Huazhong University of Science and Technology; Wuhan, China; Department of Urology; University of Michigan; Ann Arbor, MI USA; Institute of Infection Immunology; TWINCORE/Centre for Experimental and Clinical Infection Research; Hannover, Germany

†These authors contributed equally to this work.

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Human prostate cancer frequently metastasizes to bone marrow. What defines the cellular and molecular predilection for prostate cancer to metastasize to bone marrow is not well understood. CD4+CD25+ regulatory T (Treg) cells contribute to self-tolerance and tumor immune pathology. We now show that functional Treg cells are increased in the bone marrow microenvironment in prostate cancer patients with bone metastasis, and that CXCR4/CXCL12 signaling pathway contributes to Treg cell bone marrow trafficking. Treg cells exhibit active cell cycling in the bone marrow, and bone marrow dendritic cells express high levels of receptor activator of NFkB (RANK), and promote Treg cell expansion through RANK and its ligand (RANKL) signals. Furthermore, Treg cells suppress osteoclast differentiation induced by activated T cells and M-CSF, adoptive transferred Treg cells migrate to bone marrow, and increase bone mineral intensity in the xenograft mouse models with human prostate cancer bone marrow inoculation. In vivo Treg cell depletion results in reduced bone density in tumor bearing mice. The data indicates that bone marrow Treg cells may form an immunosuppressive niche to facilitate cancer bone metastasis and contribute to bone deposition, the major bone pathology in prostate cancer patients with bone metastasis. These findings mechanistically explain why Treg cells accumulate in the bone marrow, and demonstrate a previously unappreciated role for Treg cells in patients with prostate cancer. Thus, targeting Treg cells may not only improve anti-tumor immunity, but also ameliorate bone pathology in prostate cancer patients with bone metastasis.

Introduction

Bone marrow has long been known to be a primarily hematopoietic organ. However, there has been a growing realization regarding the importance of the bone marrow in immunity. For example, long-lived, antibody-secreting plasma cells reside in bone marrow. Further, a number of reports have shown that functional memory T cells exist in bone marrow. Bone marrow can serve as a site for naïve tumor associated antigen (TAA)-specific T cell priming. Interestingly, TAA-specific T cells isolated from bone marrow in tumor bearing mice and cancer patients are functional in vitro and are able to prevent tumor growth upon being transferred to another host. These observations indicate that the TAA-specific T cells are functionally suppressed in the bone marrow. In line with this, we and others have previously shown that in the homeostatic situation, mouse bone marrow harbors high levels of functional CD4+Foxp3+ regulatory T (Treg) cells. This suggests that Treg cells may form an immune suppressive niche in bone marrow, and this niche is physiologically important to keep potential inflammation at bay in this important and unique hematopoietic organ. However, it is unknown if bone marrow Treg cell compartment is altered in cancer patients with bone metastasis, and if so, what are the underlying cellular and molecular mechanisms?

In keeping these questions in mind, we further raise the point, in addition to immunosuppression, do bone marrow Treg cells affect bone pathology mediated by tumor bone metastasis in humans? It is well known that many human cancers including
Prostate cancer frequently metastasize to the bone marrow. However, the mechanisms that account for the cellular and molecular predilection for tumors to metastasize to bone marrow are not well defined. It is generally thought that tumor cells play major roles in bone pathology induced by tumor bone metastasis. It is also unknown whether immune cells including Treg cells have an impact on bone immunopathology in prostate cancer patients with bone metastasis. In order to preliminarily address these questions, we hypothesized that tumor bone marrow environmental cells provide cellular and molecular signals for Treg cell accumulation, and that high levels of Treg cells contribute to bone immunopathology in tumor bone marrow metastasis. We tested these hypotheses in patients with prostate cancer and in animal models. Our results demonstrate that high levels of Treg cells accumulate in the bone marrow in prostate cancer patients with bone metastasis, and the interaction between dendritic cells (DCs) and Treg cells promote Treg expansion, and in turn Treg cells suppress osteoclast differentiation and function, contribute to bone deposition, the predominant pathology of cancer bone metastasis.

**Results**

High levels of functional Treg cells in prostate cancer associated bone marrow. Prostate cancer frequently metastasizes to bone marrow. Recent reports suggest that bone marrow is a site for important T cell events. We previously observed high levels of Treg cells in normal bone marrow. We now examined the Treg compartment in patients with prostate cancer. We first showed that the fraction of Treg cells in CD4+ T cells was significantly higher in bone marrow in patients with prostate cancer without bone metastasis than that in normal blood and blood from patients with prostate cancer (n = 8, p < 0.01 for each). Interestingly, the levels of Treg cells were significantly higher in bone marrow from patients with prostate cancer bone marrow metastasis (37 ± 11%, n = 6, p < 0.001) than that in the bone marrow in prostate cancer patients without bone marrow metastasis (18 ± 8%, n = 6) (Fig. 1A and B). Thus, high levels of Treg cells are found in prostate cancer bone marrow metastasis.

We further tested if Treg cells associated with prostate cancer bone metastasis were functional. To this end, we employed a...
well-established assay that assesses the ability of Treg cells to mediate T cell suppression.\textsuperscript{12-15} We showed that bone marrow Treg cells from patients with prostate cancer inhibited T cell proliferation in a dose dependent manner (Fig. 1C). Bone marrow Treg cells also suppressed IFNγ and IL-2 production of T cells (Fig. 1D and E) (n = 5, \( \ast p < 0.01 \)). Thus, Treg cells in bone marrow in patients with prostate cancer are functional regulatory T cells.

**Treg cells migrate toward bone marrow via CXCR4/CXCL12 signaling pathway.** We next examined why Treg cells were accumulated in bone marrow associated with prostate cancer. The first possibility is that Treg cells efficiently traffic to bone marrow in patients with prostate cancer. We showed that Treg cells isolated from bone marrow associated with prostate cancer migrated toward human bone marrow fluid (Fig. 2A), and anti-human CXCR4 significantly decreased this migration (Fig. 2A). Further, we showed that human bone marrow produced a high level of CXCL12, the ligand for CXCR4. The levels of CXCL12 were higher in prostate cancer patients with bone marrow metastasis than normal donors (Fig. 2B). In further support, variable expression levels of several common chemokine receptors including CCR2, CCR4, CCR5, CCR7 and CXCR2 were detected in the bone marrow Treg cells, however, these Treg cells expressed high levels of CXCR4 (Fig. 2C). Therefore, the data suggests that Treg cells migrate to prostate cancer associated bone marrow by CXCR4/CXCL12 signaling pathway.

**Treg cells actively expand in tumor associated bone marrow.** After examining the possibility of Treg cell bone marrow trafficking, we further analyzed whether Treg cells were actively expanded in the tumor associated bone marrow in patients with prostate cancer. We showed that normal bone marrow Treg cells contained up to 7% Ki67 expressing cells whereas there were 1\% Ki67\textsuperscript{+} Treg cells in peripheral blood. Interestingly, there were 32\% Ki67\textsuperscript{+} bone marrow Treg cells in patients with prostate cancers (Fig. 3A and B). In accord with this, the expression of multiple cyclin genes was higher in bone marrow Treg cells than conventional T cells (Fig. 3C). On the contrary, the expression of multiple CDK inhibitors was lower in bone marrow Treg cells than conventional T cells (Fig. 3D). The data suggests that Treg cells in the tumor associated bone marrow selectively experience a pathological expansion.

**RANK\textsuperscript{+} DCs induce Treg cell expansion in tumor associated bone marrow.** We further investigated the cellular mechanism by which Treg cells were expanding in the tumor associated bone marrow. We hypothesized that tumor associated DCs might induce Treg cells expansion. We sorted blood Treg cells

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**Figure 2.** Treg migrate toward bone marrow through CXCR4/CXCL12. (A) Bone marrow Treg were subject to migrating with normal human bone marrow fluid or human bone marrow fluid from patients with bone marrow metastasis. Anti-CXCR4 and isotype were added in the migration assay (n = 6; \( \ast p < 0.01 \); compared with medium or anti-CXCR4). (B) Bone marrow expressed high level of CXCL12. CXCL12 was measured by ELISA in bone marrow fluid and blood from normal donors (n = 6) and patients with prostate cancer (n = 5). Results are expressed as mean ± SD \( \ast p < 0.01 \). (C) Bone marrow Treg cells expressed high levels of CXCR4. Bone marrow cells were stained with anti-CCR2, CCR4, CCR5, CCR7, CXCR2 and CXCR4 and Treg cell markers, and analyzed with LSR II. The chemokine receptor expression was determined by gating on CD3\textsuperscript{+}CD4\textsuperscript{+}Foxp3\textsuperscript{+} cells. One of 6 representatives is shown.
and cocultured with DCs from different sources. We showed that regardless of their source, DCs efficiently induced Treg cell expansion. However, tumor associated bone marrow DCs were superior to inducing Treg cell expansion as compared with DCs from normal bone marrow and blood (Fig. 4A). Interestingly, tumor bone marrow-associated DCs highly expressed RANK. DCs from normal blood or bone marrow without tumor in the bone marrow expressed little RANK (Fig. 4B). These DCs expressed similar levels of CD40 (Fig. 4B). To study the effects of RANK⁺ DCs on Treg cell expansion, we sorted RANK⁺ and RANK⁻ DCs from tumor associated bone marrow, and cultured with Treg cells. We observed that RANK⁺ and RANK⁻ DCs induced Treg cell expansion, however, RANK⁺ DCs were more efficient than RANK⁻ DCs to induce Treg cell expansion (Fig. 4C). Furthermore, blocking RANK/RANKL signaling pathway with recombinant osteoprotegerin (OPG) ablated the stimulatory effects of RANK⁺ DCs on Treg cell expansion (Fig. 4C). These observations suggest that RANK⁺ DCs may be responsible for Treg cell expansion in patients with prostate cancer.

Treg cells suppress osteoclast differentiation and function. In addition to immune suppression (Fig. 1), we hypothesized that high levels of Treg cells in the tumor associated bone marrow might affect bone pathology in prostate cancer. To test this, we sorted Treg cells and tested their potential roles in osteoclast differentiation induced by recombinant RANKL and M-CSF. As expected, Treg cells reduced the numbers of tartrate-resistant acid phosphatase (TRAP)-positive cells induced by RANKL (Fig. 5A and B) and M-CSF in a dose dependent manner (Fig. 5C). As activated CD8⁺ T cells were observed in bone marrow in patients with cancer,³⁶ we further tested if Treg cells affected T cell-mediated osteoclast differentiation. We observed that TRAP⁺ cells were increased in CD8⁺ T cell coculture as compared with no T cells. Treg cells reduced the numbers of TRAP⁺ cells induced by CD8⁺ T cells in a dose dependent manner (Fig. 5D). The data indicates that Treg cells suppress osteoclast differentiation.
Treg cells increase bone formation in tumor bearing mice. We tested the effects of Treg cells in vivo on bone pathology in our established human prostate cancer chimeric model. Human prostate cancer cells PC-3 were inoculated into tibia of NOD-SCID mice by intratibial injection. Tumor bone establishment was examined with both in vivo bioluminescence and radiographic imaging. Activated Treg cells were transfused into the mice by intravenous injection seven days after the establishment of bone metastasis. We showed that Treg cells increased bone mineral density (BMD) (Fig. 6A), bone mineral content (BMC) (Fig. 6B) and ameliorated bone destruction of tibial trabeculae (Fig. 6C) compared with control.

We further investigated the role of Treg cells in bone pathology in immune competent mice. To this end, RM1 tumor was initially established in the Bacterial artificial chromosome (BAC)-transgenic depletion of regulatory T cell (DREG) mouse model. Then, we injected diphtheria toxin to deplete Treg cells. Treg cells were efficiently depleted on day two, and were gradually recovered on day ten in different organs (Fig. S1). Interestingly, the levels of bone marrow Treg cells remained low on day 17 (Fig. S1). We measured BMD and BMC on day 15. We observed that Treg cell depletion resulted in partial but significant reduction of BMD (Fig. 6D) and BMC (Fig. 6E). Altogether, the data indicates that Treg cells may suppress osteoclast differentiation or function in vivo in immune competent mice.

Discussion

In this report, we observed active Treg cell recruitment and expansion in bone marrow of prostate cancer patients with bone metastasis. Furthermore, the bone marrow Treg cells tilt the balance between osteoclast and osteoblast activity, which potentially contributes to osteoblastic bone lesions that characterize prostate cancer. Finally, we have defined that CXCR4/CXCL12 and RANK/RANKL are crucial molecular signaling pathways for Treg cell bone marrow trafficking and expansion, respectively.

Several immune suppressive elements including Treg cells form immunosuppressive networks in the tumor microenvironment. We have previously demonstrated that under homeostatic situation, the levels of Treg cells in bone marrow are relatively high in healthy human beings as compared with peripheral blood and lymph nodes. This observation was confirmed in a FOXP3 bicistronic reporter knock-in mouse model. However, it was unknown if and how bone marrow Treg cells are altered in pathological scenarios. We have now observed higher levels of Treg cells in bone marrow in prostate cancer patients with bone metastasis as compared with healthy donors and patients without bone metastasis. Interestingly, prostate cancer cells and bone marrow stromal cells express high levels of CXCL12 and bone marrow Treg cells express CXCR4, and efficiently migrate toward bone marrow through CXCR4/CXCL12 signaling pathway. That activated, but not resting
Treg cells express high amount of CXCR4, indicates that Treg cells may be activated in the tumor and efficiently traffic to bone marrow, where they exert immune suppression and that immunologically facilitates tumor bone marrow metastasis.

Treg cells in bone marrow with prostate cancer metastasis express high levels of Ki67. This indicates that tumor-associated bone marrow is an organ where Treg cells actively expand. Although active bone marrow trafficking may be one of the reasons that Treg cells accumulate in bone marrow of patients with prostate cancer, extensive Treg cell expansion is an additional cause. We have determined that CXCL12/CXCR4 signaling pathway is responsible for Treg bone marrow migration and we have further explored cellular and molecular mechanisms governing Treg cell expansion in bone marrow. We demonstrated that tumor associated DCs, but not the control counterparts, induce Treg cell expansion. Interestingly, DCs from bone marrow with metastatic tumor selectively and highly expressed RANK and blockade of RANK/RANKL signaling pathway disables the effects of DC-mediated Treg cell expansion. Although IL-2 induces Treg cell expansion and TGFβ3 promotes Treg cell conversion from naïve T cells, our data indicates that the interaction between DCs and Treg cells through RANK/RANKL signaling pathway is an additional signal crucial for Treg cell expansion in specific pathological environments including prostate cancer. In support of our human studies in cancer, it has been reported that RANKL and RANK signals are implicated in Treg cell expansion in mouse models with diabetes and UV-induced immune suppression. Taken together, to ensure Treg cell bone marrow accumulation and function, bone marrow environmental cells, such as RANK+ DCs provide specific molecular signals for Treg cell expansion and in turn Treg cells mediate immune suppression, and contribute to tumor bone metastasis. Our studies focused on prostate cancer, however, it would also be interesting to examine the relationship between tumor bone metastasis and Treg cells in other human cancer settings including breast cancer.

The formation and remodeling of bone is a complex physiological process relying on a strict balance between the resorptive activity of osteoclasts and the synthesis of bone matrix by osteoblasts. RANKL/RANK is an important molecular signal pathway controlling bone remodeling and is essential for the development and activation of bone-resorbing osteoclasts. Normal bone homeostasis is achieved by a balance between the bone resorbing effects of RANKL and its natural decoy receptor OPG. Inhibition of RANKL activity with either OPG or a...
soluble RANK can inhibit prostate cancer growth including in bone.\textsuperscript{19} RANKL antibody (Denosumab) was recently approved to inhibit skeletal-related events in prostate cancer.\textsuperscript{38,39} The function of RANKL/RANK interactions outside the bone cells and tumor cells is not well defined in the context of prostate cancer. Activated T cells produce RANKL and can directly trigger osteoclastogenesis in vitro through RANK, while systemic activation of T cells in vivo results in a RANKL-mediated increase in osteoclastogenesis and bone loss.\textsuperscript{40} Although RANKL/RANK pathway is involved in Treg cell expansion in the cancer metastasis to bone marrow, we show that Treg cells inhibit osteoclast differentiation mediated by activated T cells or M-CSF and RANKL, and directly increase bone mineral density in prostate cancer. This is consistent with the observation in mouse autoimmune disease models.\textsuperscript{41,42} It remains to be defined how Treg cells do so in vivo in patients with prostate cancer. It is generally thought that prostate cancer cells play a major role in bone pathology in prostate cancer patients with bone metastasis. Our results indicate that Treg cells directly and indirectly suppress osteoclast differentiation and function, contribute to bone deposition, the important pathology of cancer bone metastasis. Given the high amount of Treg cells in prostate cancer associated bone marrow, our data provides important novel insight into osteoimmunology. As CXCR4/CXCL12 and RANKL/RANK signaling pathways are involved in immune and tumor pathology in prostate cancer bone metastasis, it is suggested that the combinatorial blockade of these two signaling pathways would be a valid option to treat patients with prostate cancer, and to ameliorate prostate cancer bone metastasis.

In summary, bone marrow in patients with prostate cancer harbors a high prevalence of functional Treg cells due to active recruitment and expansion. Treg cells interact with bone environmental cells, and this interaction provides an immune and biological environment that could favor tumor retention, growth and invasion. Thus, bone marrow Treg cells contribute to tumor bone metastatic pathology. Targeting Treg cell bone marrow recruitment and expansion may be therapeutically meaningful to treat cancer patients with bone metastasis.

**Patients and Materials and Methods**

**Human subjects.** Human bone marrow aspiration was obtained from the posterior iliac crest based on standard clinical procedure (http://www.mayoclinic.com/health/bone-marrow), and was placed in an anticoagulant tube. Certain amounts of bone marrow aspirates were used to make the appropriate slides for
pathology. Most of the bone marrow aspirates were frozen in -80°C for further functional experiments as we described previously.\(^9\) The cellular particles were diluted into single cell suspension with flow cytometry buffer for phenotyping and functional experiments as we described.\(^9\) Peripheral blood was from commercial Buffy coat cells. Donors gave written, informed consent. The study was approved by the Local Institutional Review Boards.

**Mice.** Bacterial artificial chromosome (BAC)-transgenic depletion of regulatory T cell (DEREG) mice on the C57BL/6 background were described previously\(^41\) and kept in specific pathogen-free conditions. NOD.Scid mice and wild type C57BL/6 mice were purchased from The Jackson Laboratory. Animal protocol was approved by the Unit for Laboratory Animal Medicine of University of Michigan and was conducted in accordance with the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals.

**Cell lines.** Human prostate cancer cell line PC-3 and murine prostate cancer cell line RM1\(^43\) were used in this study. PC-3 cells were stably transfected with a luc reporter vector which contains a constitutively active promoter driving luciferase expression.\(^43,44\) PC-3 and RM1 cells were cultured in vitro in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100\(\mu\)g/ml streptomycin and regularly passaged by trypsinization.

**Flow cytometry analysis.** T cells were first stained extracellularly with specific antibodies against human CD3 and CD4 (BD Biosciences), then were fixed and permeabilized with Perm/Fix solution (eBioscience) and finally were stained intracellularly with anti-Ki67, anti-IL-2, anti-IFN\(\gamma\) (BD Biosciences) and anti-Foxp3 antibodies (eBioscience). Human dendritic cells were stained with lineage markers, anti-CD11c and anti-RANK antibodies (R&D System). Samples were acquired on a LSR II (BD Biosciences) and data were analyzed with DIVA software (BD Biosciences).

**T cell proliferation and cytokine expression.** Primary T cells were stimulated with 2.5 \(\mu\)g/ml anti-CD3 and 1.25 \(\mu\)g/ml anti-CD28 monoclonal antibody (BD Biosciences) for 4 d in the presence of different concentrations of Treg cells. T cell proliferation was defined by thymidine incorporation on day 3. T cell cytokines were determined by LSR II.

**DC and Treg cell coculture.** Lin CD11c\(^+\) DCs were cultured with sorted CD4\(^+\)CD25\(^{high}\) T cells\(^45,46\) in the presence of 2.5 \(\mu\)g/ml anti-CD3 (BD Biosciences) and 5 ng/ml IL-2 (R&D System) for 4 d. T cell phenotype was determined by LSR II. The absolute numbers of Foxp3\(^+\) cells were recorded.

**Migration assay.** Migration was assessed as we described using human CD4\(^+\)CD25\(^{high}\) Treg cells (5–20 \(\times\) 10\(^4\)).\(^44,45\) Treg cells were induced to migrate with 100 ng/ml recombinant human CXCL12 (R&D System), or human bone marrow fluid. Treg cells were incubated with 500 ng/ml mouse anti-human-CXCR4 (R&D System) for 2 h as indicated. Identity of migrating Treg cells was further confirmed by flow cytometry analysis for CD3, CD4 and Foxp3 expression. Migration was expressed as a percentage of migrated cells after subtracting the spontaneous migration (Migration index).\(^44,45\)

**Quantitative real-time PCR and ELISA.** General quantitative real-time PCR was done as described.\(^45,47\) The expression of detected genes was calculated as the relative expression to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The information of the primers was listed in Table S1. CXCL12 protein in the bone marrow fluids was detected by ELISA (R&D System).

**Osteoclast differentiation assay.** Osteoclast differentiation assay was performed as we described.\(^49\) Briefly, bone marrow cells (BMCs) were obtained from C57BL/6 mice by flushing the medullary cavities ofibia and femur of the bilateral hind limbs with Dulbecco modified Eagle's medium (DMEM). These cells were cultured with 10 ng/ml M-CSF (R&D System), 50 ng/ml recombinant soluble RANKL (PeproTech) for 3 d in the presence of 20 ng/ml recombinant mouse IL-2 (R&D System). Different concentrations of CD8\(^+\) T cells or Treg cells (1 \(\times\) 10\(^4\)/ml) were pre-activated with 2.5 \(\mu\)g/ml anti-CD3 and 1.25 \(\mu\)g/ml anti-CD28 monoclonal mouse antibodies (BD Biosciences) for 12 h as we described,\(^48\) and then added into the BMCs culture for 3 d. The resultant cells were evaluated in quadruplicates. Osteoclast-like cells were identified as tartrate-resistant acid phosphatase (TRAP)-positive multinucleated (> 3 nuclei) cells with leukocyte acid phosphatase kit (Sigma) as described.\(^49,49\)

**In vivo bioluminescent imaging and radiography.** In vivo Bioluminescent Imaging was performed with an IVIS Spectrum imaging system (Xenogen Corporation, Alameda, CA) and high-resolution radiographic images of mice were obtained using a Faxitron laboratory radiography system LX-60 (Faxitron X-ray Corporation, Wheeling, IL) at 30 KVp for 10 sec.\(^44,44\)

**Human chimeric model.** The stable transfectant PC-3 cells used in in vivo study were first tested in vitro by adding 20 \(\mu\)l luciferin (40 ng/ml) into the 96-well plate. These PC-3 cells (5 \(\times\) 10\(^3\)) were intratibially injected into 4–5-week-old, male NOD.Scid mice.\(^46–48\) Tumor bone establishment was examined with both the in vivo bioluminescence intensity on a cryogenically cooled imaging system (Xenogen Corporation, Alameda, CA) coupled to a data acquisition computer and radiographic imaging. Once tumors were well established in the bone, activated Treg cells (6 \(\times\) 10\(^4\))\(^45,47\) were injected intravenously into mice on day 7 after human prostate cancer inoculation. On day 25, all the mice were sacrificed and tibiae were harvested and fixed in 10% formalin. BMD and BMC of tibial trabeculae were measured on an Eclipse Peripheral Denta Scanner (Norland Medical Systems).\(^44\)

**In vivo treg depletion.** DEREG mice were injected intratibially with RM1 cells into the right tibia as described.\(^44\) Mice were given 1 \(\mu\)g diphtheria toxin (DT) (Merck) diluted in 100\(\mu\)l endotoxin-free PBS or 100 \(\mu\)l endotoxin-free PBS alone by intraperitoneal injection on day 1, 3 and 5. DT treatment efficiently reduced Treg cells in bone marrow (Fig. S1).\(^50\) All the mice were sacrificed on day 15 and tibiae were fixed in 10% formalin. BMD and BMC were measured.

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**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.
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