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Editor's Summary

A Bone to Pick with the Immune System

School children are often taught that bones are static, providing support for the body. However, bone is actually a very dynamic tissue, constantly being built up and broken down. When this process is out of balance, for example, with excessive resorption, bone disease such as osteoporosis or fracture occurs. Now, Bozec *et al.* describe a process by which the immune system helps to control bone resorption.

The authors found that the immune costimulatory molecules CD80/86 negatively regulate generation of bone-resorbing osteoclasts. Mice deficient in these molecules were osteopenic because of increased osteoclast differentiation. Mechanistically, CD80/86 stimulation resulted in activation of an enzyme—indoleamine 2,3-dioxygenase (IDO)—in osteoclast precursors, which degraded tryptophan and promoted osteoclast apoptosis. The authors then looked in humans that had been treated with either ipilimumab (a blocking antibody to CTLA-4, which binds CD80/86) or abatacept (a CTLA-4–Ig fusion protein). Consistent with their mouse data, they found that ipilimumab increased whereas abatacept reduced the frequency of osteoclasts. These data suggest that immune costimulatory molecules can regulate bone resorption and highlight potential skeletal effects of immune costimulation–targeting therapies.

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BONE DISEASE

T Cell Costimulation Molecules CD80/86 Inhibit Osteoclast Differentiation by Inducing the IDO/Tryptophan Pathway

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Bone resorption is seminal for the physiological remodeling of bone during life. However, this process needs to be strictly controlled; excessive bone resorption results in pathologic bone loss, osteoporosis, and fracture. We describe a control mechanism of bone resorption by the adaptive immune system. CD80/86, a pair of molecules expressed by antigen-presenting cells and involved in T cell costimulation, act as negative regulator for the generation of bone-resorbing osteoclasts. CD80/86-deficient mice were osteopenic because of increased osteoclast differentiation. CD80/86-deficient osteoclasts escaped physiological inhibition by CTLA-4 or regulatory T cells. Mechanistically, engagement of CD80/86 by CTLA-4 induced activation of the enzyme indoleamine 2,3-dioxygenase (IDO) in osteoclast precursors, which degraded tryptophan and promoted apoptosis. Concordantly, IDO-deficient mice also showed an osteopenic bone phenotype with higher numbers of osteoclast precursors and osteoclasts. Also, IDO-deficient mononuclear cells escaped the anti-osteoclastogenic effect of CTLA-4. This molecular mechanism was also present in humans because targeting CD80/86 by abatacept, a CTLA-4–immunoglobulin fusion protein, reduced, whereas blockade of CTLA-4 by ipilimumab antibody enhanced, the frequency of peripheral osteoclast precursors and osteoclastogenesis. In summary, these data show an important role of the adaptive immune system, in particular T cell CD80/86 costimulation molecules, in the physiological regulation of bone resorption and preservation of bone mass, as well as affect the understanding of the function of current and future drugs fostering or blocking the effects of CTLA-4 in humans.

INTRODUCTION

Bone is continuously remodeled during life. This process controls skeletal growth during adolescence, adaptation of bone to physical demands in adulthood, and bone loss during aging. Bone remodeling is influenced by the number and activity of bone-degrading osteoclasts, which mobilize calcium and cleave the bone matrix (*1*, *2*). Although physiologically indispensible, osteoclast activity needs to be tightly controlled under physiological circumstances; increased bone resorption is a hallmark of pathological bone loss in humans, such as postmenopausal osteoporosis and bone loss during inflammatory disease.

One of the central concepts of immune-bone interactions, also termed osteoimmunology, is the interaction between T cells and osteoclast lineage cells. The immune system is one of the key factors influencing osteoclast activity in the body. Some of the most potent triggers for osteoclast differentiation, such as RANKL [receptor activator of nuclear factor κB (NF-κB) ligand], are expressed by T lymphocytes, thereby linking the adaptive immune system to bone resorption (*3*–*5*). Furthermore, certain mediators synthesized by T lymphocytes, like interleukin-17 (IL-17) and interferon-γ (IFN-γ), are among the most potent cytokine modulators for osteoclast differentiation (*6*, *7*). Current concepts thus suggest that immune activation and inflammation induce the differentiation of bone-resorbing osteoclasts, which is well in accordance with the observation of rapid bone loss and the development of premature osteoporosis during inflammatory disease (*8*). Additional observations showed that osteoclast function is governed by antibodies, Fc receptors, and related molecules, which further underlines the tight link between the adaptive immune system and bone resorption in mammals (*9*–*11*). Therefore, it can be hypothesized that the crosstalk between osteoclasts and T cells shares the principles of the interaction of antigen-presenting cells (APCs) and T lymphocytes—a pivotal checkpoint determining immune activation or immune regulation. Notably, professional APCs, such as dendritic cells and macrophages, share a common cell lineage with osteoclasts (*12*, *13*). APCs provide two essential signals for T cell activation: (i) the complex of the antigen and major histocompatibility complex (MHC) molecules, which is recognized by the T cell receptor, and (ii) the binding of costimulation molecules to respective receptors on the surface of the T cells. Only the combination of both signals triggers the activation of T cells. One of the most potent costimulation molecule complexes on the surface of APCs is CD80 and CD86, which binds to CD28 expressed on T lymphocytes (*14*, *15*). This process needs a tight regulation because continuous costimulation of T cells is deleterious. Thus, surface molecules on T cells, such as CTLA-4,

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effectively block T cell costimulation by interrupting CD80/86-CD28 interactions (*16*). Furthermore CTLA-4–mediated blockade of costimulation also appears to provide a feedback signal to the APC, modulating their function (*17*).

It is as yet unknown how osteoclast lineage cells may be affected by the costimulation system in a similar manner to professional APCs. That costimulation molecules influence the activation state of not only T cells but also APCs (*17*) suggests that the crosstalk between T cells and osteoclasts may go beyond the expression of pro- and anti-osteoclastogenic cytokines (*5*, *7*, *18*, *19*). Most importantly, the engagement of CD80/86 expressed on the surface of osteoclast lineage cells may constitute a mechanism by which the differentiation state of osteoclast linage cells could be regulated in a physiological manner by the immune system.

We therefore investigated the role of CD80/86 and other molecules involved in T cell costimulation for osteoclast differentiation and bone composition by characterizing the skeletal phenotype of costimulation mutants, defining the mechanism by which costimulation molecules influence osteoclast differentiation and translating these findings into human diseases, where therapeutic modulation of costimulation blockade is currently used.

RESULTS

Bone mass is decreased and osteoclast numbers are increased in *CD80/86*$^{-/-}$ mice

Hypothesizing that molecules involved in T cell costimulation affect osteoclast differentiation and bone composition, we first searched for a bone phenotype in various genetic mutants for essential costimulation molecules. Micro–computed tomography (μCT) analysis of the tibial bones of costimulation mutants showed profound osteopenia in *CD80/86*$^{-/-}$ mice compared to littermate controls reflected by a significantly lower bone volume and lower trabecular number (Fig. 1A). The finding of substantial bone loss in *CD80/86*$^{-/-}$ mice was surprising considering that these animals are highly protected from chronic inflammatory disease because of defective costimulation. Further histological analysis showed that the bone phenotype of *CD80/86*$^{-/-}$ mice was based on an increased osteoclast number and osteoclast-covered bone surface (Fig. 1B), suggesting enhanced osteoclastogenesis and bone resorption as the reason for the osteopenic phenotype of *CD80/86*$^{-/-}$ mice. In contrast, osteoblast counts were normal (fig. S1). To define whether bone changes are specific to *CD80/86* expression, we additionally analyzed the bone phenotype of several other mutants for costimulation molecules. Thus, mice deficient for CD28 (*CD28*$^{-/-}$), the receptor for CD80/86, as well as the costimulation molecules ICOSL and ICOS (*ICOSL*$^{-/-}$ and *ICOS*$^{-/-}$) (Fig. 1C) did not show a specific bone phenotype and also did not display any alterations in osteoclast numbers (fig. S2) compared to respective wild-type littermate control mice.

Engagement of CD80/86 inhibits osteoclast differentiation

Because histomorphometry of bones of *CD80/86*$^{-/-}$ mice showed a hyperresorptive phenotype, we suspected alterations in osteoclast differentiation associated with costimulation signals. We therefore isolated monocytes from the bone marrow of *CD80/86*$^{-/-}$ mice and respective wild-type controls and differentiated them into osteoclasts. Furthermore, we also isolated monocytes from all other costimulation molecule mutant mice shown in Fig. 1C. Monocytes from *CD80/86*$^{-/-}$ mice showed normal differentiation into osteoclasts, if cells were cultured in the presence of M-CSF (macrophage colony-stimulating factor) and RANKL (Fig. 2A). However, *CD80/86*$^{-/-}$ monocytes were completely resistant to dose-dependent inhibition of osteoclastogenesis by either soluble CTLA-4 or coculture with Foxp3-positive regulatory T cells (T_{regs}), constituting a cellular membrane-bound source of CTLA-4 (Fig. 2, A to C). These data suggest that engagement of CD80/86 by T cells and CTLA-4 is an important negative regulatory signal for osteoclast differentiation.

Furthermore, negative regulation of osteoclast differentiation was specifically dependent on CD80/86 because the absence of other molecules expressed on osteoclast precursors and involved in T cell

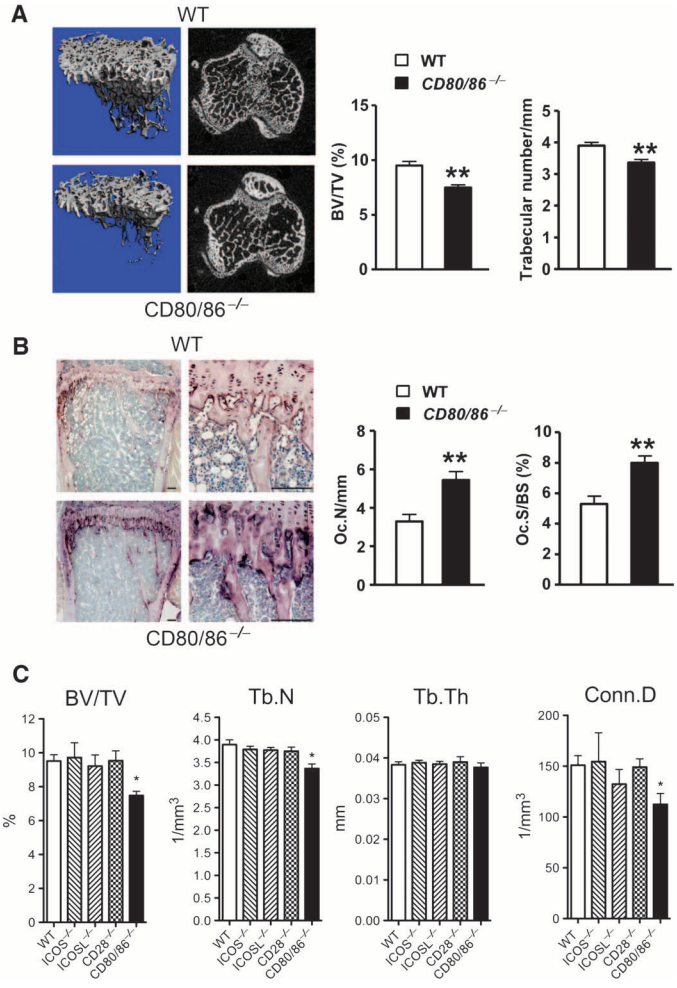

Fig. 1. Low bone mass in CD80/86-deficient mice. (**A**) Three-dimensional reconstructions of μCT scans of the proximal tibia of *CD80/86*$^{-/-}$ mice and wild-type (WT) littermate controls. Quantification of bone volume per tissue volume (BV/TV) and trabecular number (n = 10 mice per group). (**B**) Tartrate-resistant acid phosphatase (TRAP) stainings of tibial sections of *CD80/86*$^{-/-}$ mice and WT littermate controls (left: original magnification ×10, right: original magnification ×40). Quantification of osteoclast numbers (Oc.N) and osteoclast-covered surface per bone surface (Oc.S/BS) (n = 10). (**C**) Results from μCT of the tibial bone showing bone volume per tissue volume (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), and connectivity density (Conn.D) in the different costimulation mutants (n = 10).

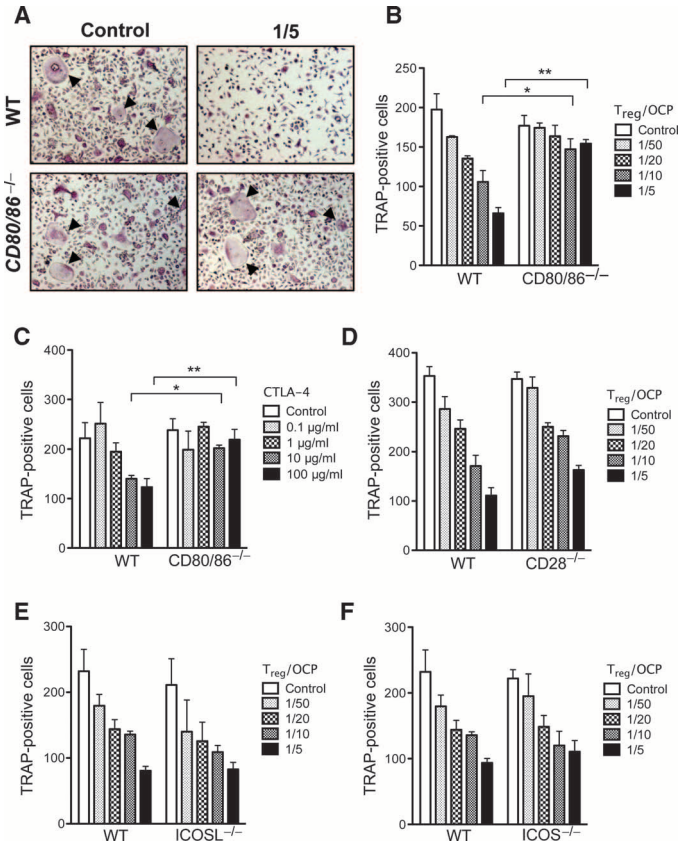

Fig. 2. Resistance of CD80/86-deficient osteoclasts to inhibition by T_{regs} and CTLA-4. (**A**) TRAP staining of osteoclast cultures from WT controls and *CD80/86*$^{-/-}$ mice cultures in the absence (control) or presence of T_{regs} (T_{reg}/osteoclast precursor ratio = 1:5). (**B**) Quantification of osteoclastogenesis in cocultures of osteoclast precursors (OCP) and various numbers of T_{regs}; control indicates coculture with activated $CD4^+$ T cells. (**C**) Quantification of osteoclastogenesis after addition of various concentrations of CTLA-4. (**D**) Cocultures of WT osteoclast precursors with T_{regs} from $CD28^{-/-}$ mice. (**E**) Cocultures of ICOSL-deficient osteoclast precursors with T_{regs} from WT mice. (**F**) Cocultures of WT osteoclast precursors with T_{regs} from $ICOS^{-/-}$ mice (n = 5). *P < 0.05; **P < 0.01.

costimulation, such as ICOSL, did not affect CTLA-4– or T_{reg}-mediated inhibition of osteoclastogenesis (Fig. 2, D to F). Also, cells derived from CD28 or ICOS mutants did not show an overt differences in osteoclast differentiation compared to wild-type controls (Fig. 2, D to F).

Indoleamine 2,3-dioxygenase expression is induced in osteoclast precursors upon challenge with CTLA-4

We next aimed to define the molecular mechanism by which engagement of CD80/86 by CTLA-4 suppresses osteoclastogenesis. CTLA-4 has been previously described to induce indoleamine 2,3-dioxygenase (IDO) expression in dendritic cells upon binding to CD80/86 (*17*). IDO plays a role in mediating the immune-suppressive effects of T_{regs} by catalyzing the metabolism of tryptophan to kynurenine and inducing apoptosis (*20*, *21*). In our experiments, IDO mRNA expression was significantly induced in osteoclast precursors upon coculture with T_{reg} or upon challenge with CTLA-4 (Fig. 3A). In contrast, IDO mRNA expression was not induced by CTLA-4 in CD80/86-deficient cells (Fig. 3B). Furthermore, IDO protein expression robustly increased in osteoclast precursors after challenge with CTLA-4, whereas only very moderate effect was observed in CD80/86-deficient cells (Fig. 3B), suggesting that CD80/86 engagement by CTLA-4 leads to induction of IDO mRNA and protein expression in osteoclast precursor cells.

CTLA-4 induces tryptophan degradation and apoptosis of osteoclast precursor cells

To address whether alterations in IDO expression by CTLA-4 are also linked to metabolic changes in osteoclast lineage cells, we made use of the enzymatic activity of IDO, which degrades the amino acid tryptophan, leading to increased levels of its metabolite kynurenine and thereby altering the ratio between kynurenine and tryptophan (*22*, *23*). Indeed, mass spectrometry–based measurement of kynurenine and tryptophan in the cell culture supernatants showed an increase of the kynurenine/tryptophan ratio after challenge of osteoclast precursors with either T_{regs} or CTLA-4 (Fig. 3C). Furthermore, induction of IDO activity and tryptophan catabolism was associated with enhanced apoptosis in osteoclast precursors as shown by enhanced staining with annexin V as well as increased expression of cleaved caspase-3 (Fig. 3D and fig. S3). CTLA-4–mediated induction of IDO activity and increased apoptosis were not observed in CD80/86-deficient osteoclast precursors (Fig. 3D). In contrast, no significant changes in osteoclast precursor proliferation of wild-type and CD80/86-deficient mice were observed (fig. S4). Thus, these findings suggest that engagement of CD80/86 controls the pool of osteoclast lineage cells and thereby serves as checkpoint for osteoclast numbers.

CTLA-4–CD80/86–mediated IDO activation is NF-κB–dependent

We also performed specific knockdown experiments to show that CTLA-4–CD80/86–mediated apoptosis of osteoclast lineage cells indeed depends on IDO. When osteoclast precursors were transfected with small interfering RNAs (siRNAs) that efficiently suppressed CTLA-4–induced IDO expression (Fig. 3E), the suppressive effect of T_{regs} and CTLA-4 on osteoclastogenesis was neutralized as shown by complete rescue of osteoclast differentiation in the presence of CTLA-4 and partial rescue in the presence of T_{regs} (Fig. 3F). Considering the functional importance of IDO in this process, we further investigated the mechanism of its induction upon engagement of CD80/86 by CTLA-4. Previous data suggested that IDO could be activated by the noncanonical NF-κB pathway (*17*). Furthermore, we could show that CTLA-4 induces the expression of both IKKα [IκB (inhibitor of NF-κB) kinase α] and NIK (NF-κB–inducing kinase) in osteoclast precursor cells (Fig. 3G). We therefore attempted to inhibit CTLA-4–mediated IDO activation by blocking the noncanonical NF-κB pathway. Whereas IKKα-targeted siRNA did not significantly inhibit CTLA-4–induced IDO protein expression, inhibition of NIK showed a robust decrease in IDO protein expression associated with a significant reduction of IDO activity in osteoclast precursors as measured by the decrease in kynurenine/tryptophan ratio resembling tryptophan degradation (Fig. 3, H and I).

IDO-deficient (*IDO*$^{-/-}$) mice are osteopenic with high number of osteoclast precursor cells

To further support the role of IDO in controlling osteoclast numbers in vivo, we analyzed the bone phenotype of *IDO*$^{-/-}$ mice. μCT analyses revealed significant reduction of trabecular bone volume, trabecular number, and connectivity in *IDO*$^{-/-}$ mice compared to littermate

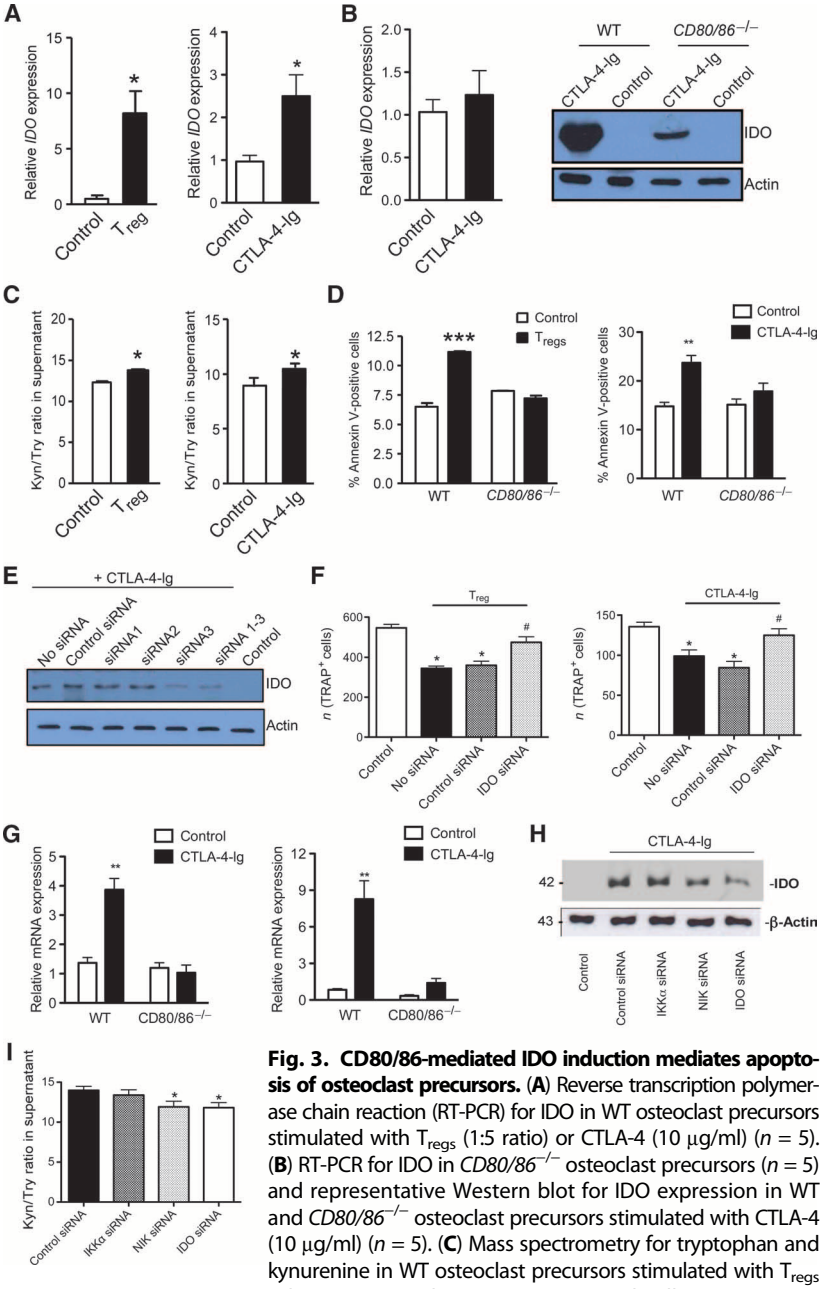

Fig. 3. CD80/86-mediated IDO induction mediates apoptosis of osteoclast precursors. (**A**) Reverse transcription polymerase chain reaction (RT-PCR) for IDO in WT osteoclast precursors stimulated with T_{regs} (1:5 ratio) or CTLA-4 (10 μg/ml) ($n = 5$). (**B**) RT-PCR for IDO in *CD80/86*$^{-/-}$ osteoclast precursors ($n = 5$) and representative Western blot for IDO expression in WT and *CD80/86*$^{-/-}$ osteoclast precursors stimulated with CTLA-4 (10 μg/ml) ($n = 5$). (**C**) Mass spectrometry for tryptophan and kynurenine in WT osteoclast precursors stimulated with T_{regs} (1:5 ratio) or CTLA-4 (10 μg/ml) ($n = 5$). (**D**) Fluorescence-activated cell sorting (FACS) analysis for apoptotic annexin V–positive osteoclast precursors after stimulating WT and *CD80/86*$^{-/-}$ osteoclast precursors stimulated with T_{reg} (1:5 ratio) or CTLA-4 (10 μg/ml) ($n = 5$). (**E**) Representative Western blot for IDO expression in WT osteoclast precursors stimulated with CTLA-4 (10 μg/ml) and exposed to three different siRNAs for IDO (siRNA 1-3). (**F**) Numbers of osteoclasts in cultures of WT osteoclast precursors stimulated with either T_{regs} (1:5 ratio) or CTLA-4 (10 μg/ml) together with control and IDO-specific siRNA (siRNA 1-3) ($n = 5$). (**G**) RT-PCR for IKKα (left) and NIK (right) in WT osteoclast precursors stimulated with CTLA-4 (10 μg/ml) ($n = 5$). (**H**) Western blot for IDO expression in WT osteoclast precursors stimulated with CTLA-4 (10 μg/ml) and exposed to control siRNA or siRNA for IKKα or NIK (siRNA 1-3). (**I**) Mass spectrometry for tryptophan and kynurenine in WT osteoclast precursors stimulated with CTLA-4 (10 μg/ml) and control siRNA or siRNA for IKKα, NIK, or IDO (siRNA 1-3) ($n = 5$).

controls (Fig. 4, A and B). When analyzing the number of osteoclast precursor cells in the spleen and the bone marrow, we found them significantly increased in *IDO*$^{-/-}$ mice compared to controls (Fig. 4C). Very similar results were obtained when analyzing frequencies of osteoclast precursor cells in the spleen and bone marrow of CD80/86$^{-/-}$ mice (fig. S5). Osteoclast precursor cells from *IDO*$^{-/-}$ mice had no higher intrinsic osteoclastogenic potential compared to cells from wild-type controls on the individual cell level (Fig. 4D). However, on the basis of the higher frequency of osteoclast precursors, the mononuclear cell fraction from the spleen of *IDO*$^{-/-}$ mice showed significantly higher osteoclastogenic potential than those from wild-type controls (Fig. 4E). Furthermore, when mononuclear cells from wild-type controls and *IDO*$^{-/-}$ mice were cultured under osteoclastogenic conditions, coculture with T_{regs} or addition of CTLA-4 led to a dose-dependent inhibition of osteoclast differentiation only in wild-type but not in IDO-deficient cells (Fig. 4F).

CTLA-4 treatment leads to reduced osteoclast precursor cell frequency and osteoclastogenesis in humans

To translate these findings into the human situation, we considered that the frequency of osteoclast precursors in the peripheral blood and, in consequence, also osteoclastogenesis may be impaired after exposure of humans to CTLA-4. Abatacept, a CTLA-4–Fc fusion protein, is approved for the treatment of rheumatoid arthritis (*24*). We therefore investigated osteoclast precursor frequency in the peripheral blood of rheumatoid arthritis patients treated with abatacept and compared it to untreated, methotrexate-treated, and tumor necrosis factor (TNF) blocker–treated rheumatoid arthritis patients as well as healthy controls. The frequency of osteoclast precursors was significantly higher in rheumatoid arthritis patients than in healthy controls (Fig. 5A). Whereas methotrexate treatment and TNF inhibition led to a slight reduction of osteoclast precursors, we found the most pronounced reduction in abatacept-treated rheumatoid arthritis patients, in whom osteoclast precursor frequency was indistinguishable from the one observed in healthy controls (Fig. 5A). Furthermore, culture of mononuclear cells from these patients under osteoclastogenic conditions revealed similar results: Rheumatoid arthritis patients showed significantly ($P < 0.01$) higher osteoclastogenic potential than healthy controls, which was slightly lower in methotrexate- and TNFi-treated rheumatoid arthritis patients and fully reversed by abatacept treatment (Fig. 5, A to C). In vitro, exposure of human osteoclast precursors to abatacept was followed by rapid down-regulated expression of osteoclast marker genes such as *c-Fos* after 30 min, *NFATc1* and *C-Fms* (CD115) after 1 hour, and *RANK* after 3 hours (Fig. 5D). Finally, we also had the opportunity to analyze some patients before exposure and 4 weeks after exposure to abatacept: Analysis of osteoclast precursor frequency in the peripheral blood

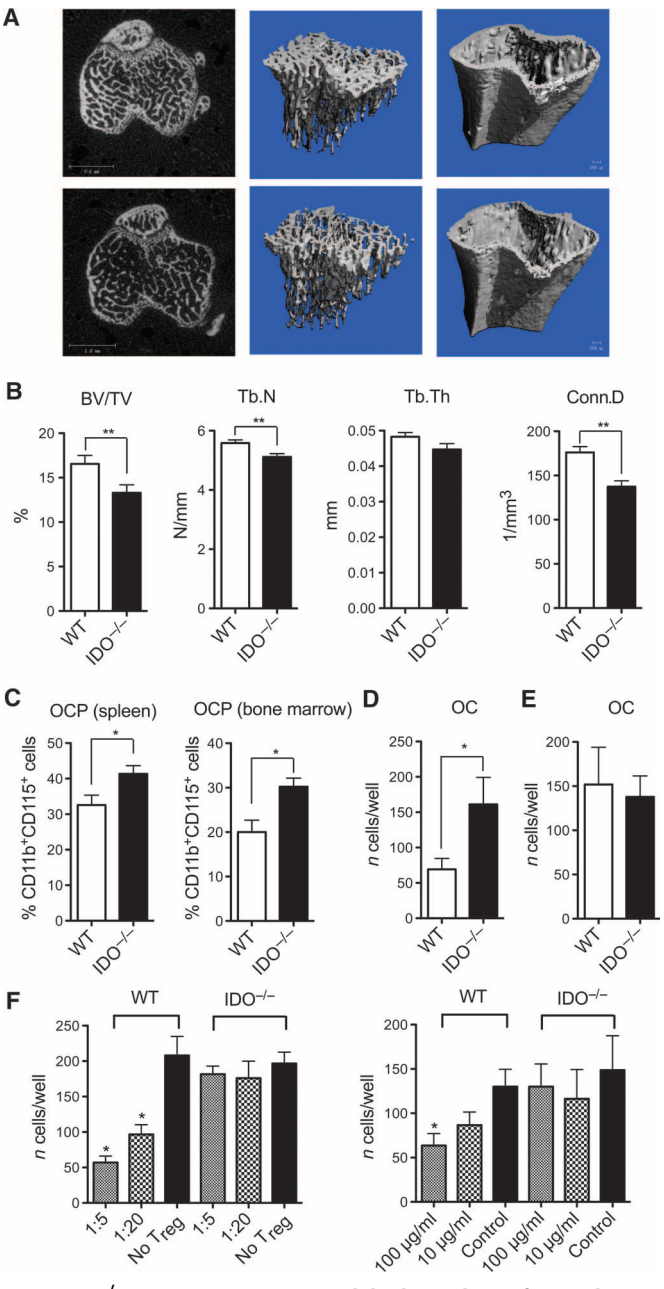

Fig. 4. *IDO*$^{-/-}$ mice are osteopenic with high numbers of osteoclast precursors and osteoclasts. (**A**) Two- and three-dimensional reconstructions of μCT scans of the proximal tibia of *IDO*$^{-/-}$ mice and WT littermate controls. (**B**) Quantification of bone volume per tissue volume (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), and connectivity density (Conn.D) (n = 10). (**C**) FACS analysis of osteoclast precursor frequency in the spleens (left panel) and the bone marrow (right panel) from *IDO*$^{-/-}$ mice and WT littermate controls (n = 5). (**D**) Osteoclast differentiation assay from whole spleen cells of *IDO*$^{-/-}$ mice and WT littermate controls showing the number of TRAP-positive multinucleated osteoclasts (n = 5). (**E**) Osteoclast differentiation assay from the same number of CD11b^{+}CD115^{+} monocytes from the spleens of *IDO*$^{-/-}$ mice and WT littermate controls (n = 5). (**F**) Numbers of osteoclasts in cultures of *IDO*$^{-/-}$ and WT osteoclast precursors stimulated with either T_{regs} or CTA-4 (n = 5).

revealed a significant decrease 4 weeks after abatacept exposure, and similar results were obtained for osteoclast differentiation potential after culturing cells under osteoclastogenic conditions (Fig. 5E).

Blockade of CTLA-4 increases osteoclast precursors and osteoclastogenesis in humans

Having shown that CTLA-4 treatment reduces osteoclast precursor frequency and the osteoclastogenic potential of PBMCs in humans, we questioned whether blockade of CTLA-4 might yield the opposite result. We made use of the opportunity that aside from administration of

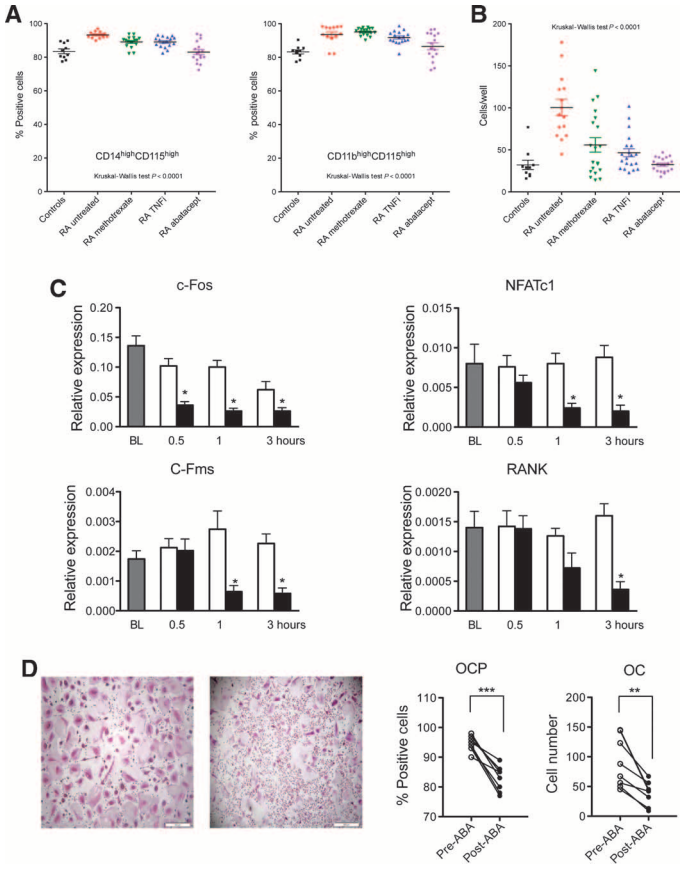

Fig. 5. CTLA-4 regulates human osteoclast precursor numbers and osteoclastogenesis. (**A**) Frequency of circulating CD14^{+}/CD115^{+} osteoclast precursors (left) and circulating CD11b^{+}/CD115^{+} osteoclast precursors (right) among peripheral monocytes in healthy individuals (black; n = 10) and untreated (red; n = 15), methotrexate-treated (green; n = 17), TNF inhibitor (TNFi)–treated (blue; n = 17), and abatacept-treated (CTLA-4, purple; n = 17) rheumatoid arthritis (RA) patients. (**B**) Number of TRAP-positive multinucleated osteoclasts after culture of peripheral blood mononuclear cells (PBMCs) from healthy individuals (black; n = 10) and untreated (red; n = 15), methotrexate-treated (green; n = 17), TNFi-treated (blue; n = 17), and abatacept-treated (CTLA-4, purple; n = 17) rheumatoid arthritis patients with M-CSF and RANKL for 2 weeks. (**C**) Real-time PCR of osteoclast precursors stimulated with M-CSF and RANKL in the absence (white) or presence (black) of abatacept (CTLA-4). BL, baseline (n = 3). (**D**) Left: Representative TRAP stainings of osteoclasts from rheumatoid arthritis patients before and after treatment with abatacept (CTLA-4). Right: Quantification of osteoclast precursor cell frequency and osteoclast numbers before and after abatacept (CTLA-4) (n = 7).

CTLA-4, also inhibition of CTLA-4 is in clinical use. Thus, ipilimumab, a neutralizing antibody against CTLA-4, fosters the immune response and is used for the treatment of advanced stages of melanoma-type skin cancer (*25*). Ipilimumab-treated patients had significantly higher frequency of osteoclast precursors than controls, contrasting the effects of abatacept (Fig. 6A). In osteoclast differentiation cultures, no increase in osteoclastogenesis was found in ipilimumab- treated patients when adjusting for the numbers of osteoclast precursors (Fig. 6B). However, when assessing the overall osteoclast differentiation potential of PBMCs, ipilimumab-treated patients showed an almost twofold increase in osteoclastogenesis, reflecting the higher number of circulating osteoclast precursors in these patients (Fig. 6B). Furthermore, when assessing osteoclast precursor frequency and osteoclastogenesis before and after exposure to ipilimumab in the same patients, we found both parameters significantly increased, suggesting that blockade of CTLA-4 leads to increased osteoclastogenic potential in humans in vivo (Fig. 6C).

DISCUSSION

Here, we show that classical immune costimulation pathway regulates osteoclast differentiation in mouse and human. CD80/86, a pair of costimulation molecules, mediating binding of APCs to CD28 on T cells, also represents a strong inhibitory signal for osteoclast differentiation and thereby protects the body from uncontrolled bone resorption. Engagement of CD80/86 by the regulatory molecule CTLA-4 induces the expression of the enzyme IDO in osteoclast lineage cells, which is the rate-limiting enzyme of tryptophan catabolism and involved in cell apoptosis. Physiological control of osteoclast differentiation by CD80/86 thereby appears to have direct relevance in medicine because intervention strategies blocking and fostering CTLA-4–CD80/86 interactions are currently used for the treatment of inflammatory diseases, transplant rejection, and cancer (*24*, *25*).

The crosstalk between monocyte lineage cells, such as macrophages and dendritic cells, and T cells is one of the central principles of immune activation and regulation. During this process, monocyte lineage cells provide essential signals to activate T cells: (i) the antigen in complex with MHC molecules for T cell receptor recognition and (ii) costimulatory signals, which are recognized by specific surface receptors on T lymphocytes. This process is not unidirectional because signals derived from the T cell lineage, like CTLA-4, provide a negative feedback loop stopping stimulation of T cells by APC. Whether osteoclastogenesis and bone resorption are affected by this costimulation crosstalk, however, has not been recognized to date. Nonetheless, several lines of evidence support that osteoclasts share intensive communication with T lymphocytes like professional APCs. Thus, osteoclasts express MHC molecules and can serve as APCs (*26*). Furthermore, osteoclasts share surface receptors and receptor-ligand interactions with other monocytic lineages, particularly with dendritic cells, which share common ancestry with osteoclasts (*12*, *13*). One example is RANK, which is expressed on both dendritic cells and osteoclasts and allows respective cell responses, namely, IL-12 expression and osteoclast differentiation, upon engagement of T cell–derived RANKL (*19*). Finally, the basic principle of osteoimmunology is built upon the observation that T cells influence osteoclast differentiation and activation not only by RANKL but also by T cell–derived cytokines such as IL-17 acting as activation signal and IFN-γ as suppressive signal (*6*, *7*).

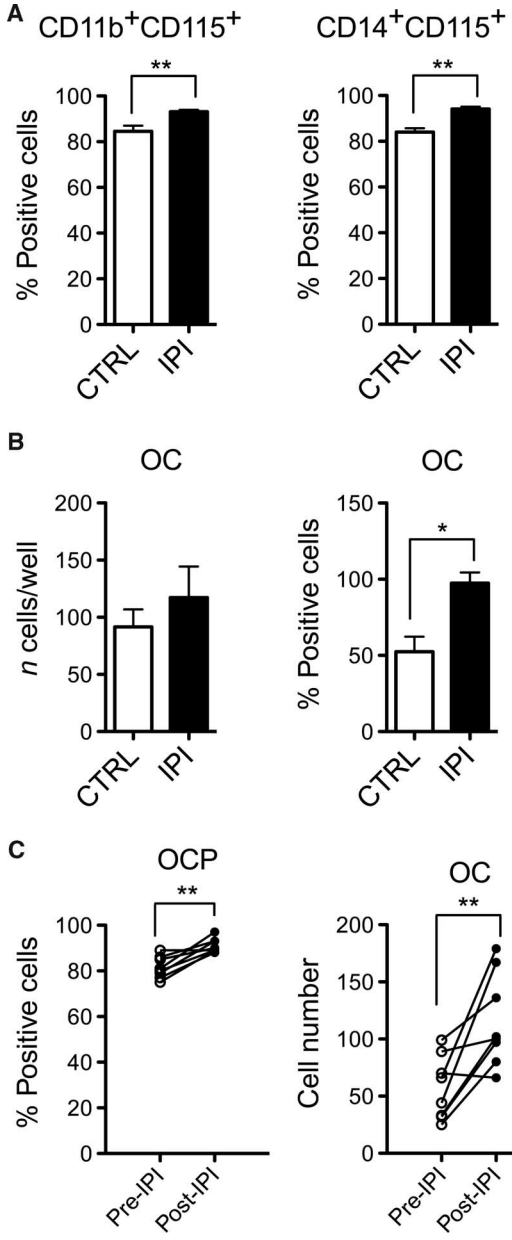

Fig. 6. Blockade of CTLA-4 in humans increases human osteoclast precursor number and osteoclastogenesis. (**A**) Frequency of circulating $CD11b^+CD115^+$ osteoclast precursors (left) and circulating $CD14^+CD115^+$ osteoclast precursors (right) among peripheral monocytes in melanoma patients without ipilimumab (IPI) treatment (white; $n = 10$) and those receiving ipilimumab treatment (anti–CTLA-4; $n = 10$). (**B**) Number of TRAP-positive multinucleated osteoclasts after culture of PBMCs from melanoma patients without ipilimumab treatment (white; $n = 10$) and those receiving ipilimumab treatment ($n = 10$). (**C**) Osteoclast precursor cell frequency and osteoclast numbers before and after ipilimumab treatment (anti–CTLA-4) ($n = 8$).

We and others have previously shown that T_{regs} are potent suppressor of osteoclast differentiation, suggesting that, in addition to IFN-γ, other T cell–derived signals inhibit osteoclast differentiation (*27*–*30*).

The observation that cell-cell contact is important for suppression of osteoclastogenesis by T_{regs} further substantiates the concept that cell-bound signals involved in monocyte–T cell interaction modulate osteoclast differentiation and bone resorption (*29*). In addition, CTLA-4, which is preferentially expressed on T_{regs} and a potent negative regulator of costimulation, has shown to bind to osteoclast precursors, block their differentiation into osteoclasts, and mitigate inflammatory bone loss (*31*). The mechanism, however, by which T_{regs} and CTLA-4 affect bone resorption and thereby protect from pathological bone loss remains unclear. Our observation that both genetic and pharmacological neutralization of CD80/86 completely restored osteoclast differentiation despite the presence of T_{regs} and CTLA-4 clearly pointed to an essential regulatory role of this costimulation molecule in osteoclast formation. The physiological importance of this interaction is underlined by the osteoporotic phenotype of *CD80/86*$^{-/-}$ mice, which lack the inhibitory effect of T_{regs} on the osteoclast lineage. Therefore, enhanced osteoclast formation and bone resorption are observed in vivo. This effect is not redundant, but specific for CD80/86, because the deletion of other costimulation molecules such as ICOS, ICOSL, and CD28 did not affect the suppressive effect of T_{regs} on osteoclasts and did not lead to any bone phenotype.

The central regulatory role of CD80/86 in osteoclast formation appears to be linked to the immunomodulatory enzyme IDO, which is the key enzyme degrading tryptophan. Activation of IDO and tryptophan degradation induce T cell tolerance and lead to apoptosis of T cells as well as monocytes (*20*–*23*, *32*–*35*). These effects are regulated by IDO expression in APCs such as dendritic cells (*32*). Previous data have shown not only an association between CD80/86 and IDO expression in dendritic cells but also up-regulation of IDO by CTLA-4 (*17*). We observed that exposure of osteoclasts to either T_{regs} or CTLA-4 induced not only expression but also activation of IDO, represented by the increased level of kynurenine, the major product of tryptophan catabolism. Induction of IDO in osteoclast progenitors enhanced osteoclast precursor cell apoptosis. Thereby, IDO induction and apoptosis elicited by either CTLA-4 or T_{regs} were strictly dependent on the expression of CD80/CD86 because no such effects were observed in CD80/86-deficient osteoclast precursors. Even more importantly, normal osteoclastogenesis in the presence of CTLA-4 or T_{regs} could be restored when IDO was down-regulated by specific inhibitory RNAs.

Signaling between CD80/86 and IDO appears to involve the noncanonical NF-κB pathway. NIK has been shown to play an important role in the induction of IDO expression in dendritic cells (*17*). CTLA-4 led to a robust increase in IKKα and NIK expression in osteoclast precursors. Down-regulation of NIK abolished the induction of IDO expression by CTLA-4. These data point to a more regulatory function of NIK in osteoclastogenesis, particularly in T cell–osteoclast interactions, which does not necessarily contradict previous findings that osteoclast differentiation is impaired when NIK is genetically depleted (*36*). It is rather conceivable that transient activation of NIK is required to mediate CD80/86-triggered induction of IDO, which then modulates tryptophan metabolism and triggers a proapoptotic state in the osteoclast precursors. Our in vivo data from *IDO*$^{-/-}$ mice and in vitro data from IDO-deficient cells support such concept. Thus, *IDO*$^{-/-}$ mice show an osteopenic bone phenotype associated high numbers of peripheral osteoclast precursors and enhanced osteoclast differentiation potential. Neither T_{regs} nor CTLA-4 was capable of modulating osteoclastogenesis in IDO-deficient cells, suggesting that the regulatory effect of CD80/86 on osteoclasts is exclusively mediated by IDO. Thus, IDO not only affects the communication between cells of the innate immune system and T cells but also regulates bone homeostasis.

Direct clinical implications from these findings arise because modulators of the CTLA-4–CD80/86 axis are among the most potent immunomodulatory and anticancer drugs. Both CTLA-4 agonists and antagonists are currently used for the treatment of autoimmune disease and cancer (*24*, *25*). Abatacept, a fusion protein of CTLA-4 and immunoglobulin G (IgG), is a potent drug for the treatment of rheumatoid arthritis, in which it also exerts bone-protective effects (*24*). In investigating patients with rheumatoid arthritis, we and others have demonstrated an enhanced frequency of peripheral osteoclast precursors associated with increased osteoclastogenic potential of PBMCs (*37*, *38*). Abatacept completely abrogated this increase in osteoclast precursor frequency in patients with rheumatoid arthritis as well as the pro-osteoclastogenic state of PBMCs. These findings indicate that CD80/86 also controls human osteoclastogenesis. In accordance with this concept, the bone-protective effect of abatacept in patients with rheumatoid arthritis is well documented. On the other hand, ipilimumab, a neutralizing antibody against CTLA-4, is successfully used for the treatment of malignant melanoma. Ipilimumab fosters autoimmunity and, in consequence, anticancer immunity (*25*). The effect of ipilimumab on osteoclasts was exactly the opposite of that of abatacept, further supporting the role of the CTLA-4–CD80/86 axis in human bone homeostasis in vivo.

There are a few limitations of this study: Data on bone architecture in patients treated with abatacept and ipilimumab were not available. Because of the fact that the target diseases for abatacept and ipilimumab, rheumatoid arthritis and malignant melanoma, respectively, are associated with bone loss, such studies are important but require rigorous control of confounding factors and cutting-edge bone imaging techniques. Furthermore, bone-protective effects of abatacept in vivo may not be solely based on regulating osteoclast precursor frequency but are likely to be additionally enhanced by its blocking effect on T cell activation and T cell–mediated cytokine production, which have not been studied here, but have been characterized previously (*39*).

In summary, our data show a mechanism by which the immune system protects the bone. CD80/86 and IDO regulate the number and survival of peripheral osteoclast precursors and the osteoclastogenic potential of mononuclear cells in both mice and humans. This finding extends the role of the T cell costimulation system to bone. Thus, immune regulatory processes, which determine the functional state of dendritic cells as well as T cells, also control the delicate balance of bone homeostasis by mitigating the process of osteoclast differentiation. On the basis of our data, bone homeostasis becomes even more closely related to immune homeostasis. These interactions have direct clinical consequences with respect to bone homeostasis in humans because modulators of the costimulation system are already used in the pharmacotherapy of autoimmune disease and cancer.

MATERIALS AND METHODS

Study design

All murine and human samples were analyzed in blinded fashion. The person investigating the samples was unaware of the genotype of mice or, in case of human samples, of the patient identity and treatment status. At least three independent experiments were performed for all laboratory analyses in this study. For analyzing mouse phenotypes,

littermate controls were used. For analyzing human phenotypes, consecutive patients were enrolled.

Animals

CD80/86$^{-/-}$, *IDO*$^{-/-}$, *CD28*$^{-/-}$, *ICOS*$^{-/-}$, and *ICOSL*$^{-/-}$ were maintained in C57BL/6 background in a specific pathogen–free facility. Twelve-week-old female mice were used in all the analyses. All animal experiments were performed with the agreement of the ethic local authorities. All experiments were performed using sex-matched mutant and wild-type control littermates.

Micro–computed tomography

The right tibia was prepared and stored in 70% ethanol for μCT analysis. μCT analyses were done with a commercially available desktop μCT (μCT35, Scanco Medical AG). The following acquisition parameters were used: voltage, 40 kV; x-ray current, 250 μA; exposure time, 5000 ms per 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 (Mercury) with the following histomorphometric parameters at the metaphyses of the proximal tibiae: bone volume/total volume, trabecular thickness, trabecular number, and connectivity density (*40*).

Bone histology

Mice were treated as described above, and the left tibia was used for histology. Bones were fixed overnight in 4% formalin and decalcified in EDTA (Sigma) until they were pliable. Serial paraffin sections (2 μm) were stained for TRAP (Sigma) for the detection of osteoclasts. All analyses were performed using a microscope (Nikon) equipped with a digital camera and an image analysis system for performing histomorphometry (OsteoMeasure, OsteoMetrics).

Isolation and culture of osteoclasts

For osteoclast assays, bone marrow was isolated from *CD80/86*$^{-/-}$, *CD28*$^{-/-}$, *ICOS*$^{-/-}$, *ICOSL*$^{-/-}$, and wild-type mice by flushing femoral bones with complete α-DMEM (Dulbecco's modified Eagle's medium). Osteoclast precursors were then isolated from bone marrow–derived cell suspensions using CD11b microbeads (Miltenyi Biotec) according to the manufacturer's instructions. The purity of isolated monocytes was assessed by flow cytometric analysis using CD11b-FITC (fluorescein isothiocyanate)–labeled antibodies (Miltenyi Biotec). CD11b^{+} cells were plated in flat-bottomed 96-well plates (2.5×10^5 per well) or 48-well plates (5×10^5 per well) in α-MEM (minimum essential medium) supplemented with 10% fetal calf serum, M-CSF (30 ng/ml), and RANKL (50 ng/ml) (all R&D Systems). TRAP staining was performed after 5 days, osteoclasts were identified by purple color and the presence of three or more nuclei, and osteoclast precursors were identified by purple color and the presence of one or two nuclei. To assess the effect of CTLA-4 and T_{regs} on osteoclast differentiation, cultures were performed in the presence of different doses of CTLA-4 (0.1 to 100 μg/ml) (Bristol-Myers Squibb) or various numbers of activated CD4^{+}CD25^{+}Foxp3^{+} T_{regs} (5×10^4 to 5×10^3 per well corresponding to a T cell/APC ratio of 1:5 to 1:50). T_{regs} were isolated from the spleens of sex- and age-matched wild-type mice by microbead-based Regulatory T Cell Isolation Kit (Miltenyi Biotec) according to the manufacturer's instructions. The purity and functionality of the purified cells were analyzed as previously described (*29*).

Immunoblotting

Cells from above-mentioned osteoclast cultures were lysed in Laemmli buffer (Bio-Rad), and proteins were separated by electrophoresis in 10% SDS–polyacrylamide gels and blotted onto polyvinylidene difluoride membranes (Millipore). After being blocked with 5% dry milk prepared in phosphate-buffered saline (PBS) containing 0.1% Tween 20, the membranes were incubated with monoclonal antibody against IDO (Millipore). Detection was done with peroxidase-conjugated antibodies (all Promega) and chemiluminescence reagent (ECL) (Thermo Scientific).

Real-time PCR

RNA was isolated from nonsilencing and knockdown cells using RNeasy Kit (Qiagen) according to the manufacturer's protocol. Then, complementary DNA (cDNA) was synthesized from 1 μg of RNA using iScript cDNA Synthesis RT-PCR kit (Bio-Rad) according to the user manual. The resulting cDNA was then amplified by quantitative real-time PCR using iQ SYBR Green PCR Supermix (Bio-Rad) and iCycler iQ Real-Time PCR Detection System (Bio-Rad). cDNA levels were normalized to actin. The following primers were used: *ido*, 5′-CGGACTGAGAGGACACAGGTTAC-3′ and 5′-ACACATACGCCATGGTGATGTAC-3′; *nik*, 5′-TGCGGAAAGTGGGAGATCCTGAAT-3′ and 5′-TGTACTGTTTGGACCCAGCGATGA-3′; *IKKa*, 5′-GACCGTGAACATCCTCTGACATGTG-3′ and 5′-GCTCTGGTCCTCATTTGCTTCACG-3′; actin, 5′-TAAAACGCAGCTCAGTAACAGTCCG-3′ and 5′-TGGAATCCTGTGGCATCCATGAAAC-3′.

Tryptophan/kynurenine measurement

Tryptophan and kynurenine were determined simultaneously using liquid chromatography–tandem mass spectrometry, with atmospheric pressure chemical ionization in the positive ion mode (API 4000 Q Trap, Applied Biosystems, MDS Sciex). Ten microliters of the samples and calibrators was deproteinized with sulfosalicylic acid (10%). Liquid chromatography was performed using a Chromolith column (RP-18e, 100 × 3.0 mm) (Merck) at a flow rate of 1 ml/min with 2 mM ammonium acetate/methanol (78:22, v/v; pH 2.0). The total run time was 2.5 min. Sample analysis was performed in the multiple reaction monitoring mode with a dwell time of 100 ms per channel using the following transitions for quantification (qualifier transition): mass/charge ratio (*m/z*) 205.2/187.8 (205.2/146.0) tryptophan, *m/z* 209.2/191.7 (209.2/146.0) kynurenine, *m/z* 210.2/192.8 tryptophan-d5 (internal standard).

Transfection with siRNA

Osteoclast precursors were transfected in Opti-MEM I Reduced Serum Medium (Invitrogen) with IDO-, NIK-, or IKKα-specific siRNA (Stealth Select RNAi, Invitrogen) or a negative control siRNA (Stealth RNAi Negative Control Duplexes) using Lipofectamine RNAiMAX (Invitrogen) for 6 hours according to the manufacturer's instructions. Transfection efficiency was assessed by BLOCK-iT Alexa Fluor Oligo (Invitrogen) positive control.

Flow cytometry

Isolated murine spleen cells (1×10^6) were washed with 1 ml of PBS containing 0.1% bovine serum albumin and incubated with saturating amounts of FITC- or phycoerythrin-labeled antibodies against CD11b (BD Biosciences) and CD115 (c-Fms, M-CSF receptor) (BioLegend).

Cells were also stained with isotype control antibodies and analyzed by FACSCanto flow cytometer (BD Biosciences).

Human osteoclast cultures

PBMCs were isolated from 10 ml of EDTA blood of healthy donors, rheumatoid arthritis patients, and melanoma patients using a Ficoll gradient (Lymphoflot, Bio-Rad) and incubated in 48-well plates (7.5×10^5 cells per well) in α-MEM (Gibco) with 1% penicillin-streptomycin (Gibco). After 3 hours, the cells were washed for monocyte enrichment and cultivated for 14 days in α-MEM supplemented with 10% heat-inactivated fetal bovine serum (Biochrom AG), 1% penicillin/streptomycin (Gibco), M-CSF (10 ng/ml), and submaximal concentrations of RANKL (1 ng/ml) (both PeproTech). Every 3 days, the medium was replaced with fresh medium. Osteoclast differentiation was evaluated by staining cells for TRAP using an Acid Phosphatase, Leukocyte Kit (Sigma-Aldrich) according to the manufacturer's instructions.

Human osteoclast precursors

Blood samples (20 ml) from healthy control subjects and patients with rheumatoid arthritis or malignant melanoma were collected in EDTA-containing Vaccutubes. Ficoll density gradient centrifugation was used to isolate mononuclear cells. Osteoclast precursor numbers were assessed with FACS by applying antibodies against CD11b or CD14 and the M-CSF receptor. The cells were also stained with isotype control antibodies and analyzed with a FACSCanto flow cytometer (BD Biosciences). Double-positive cells were quantified.

Human RNA expression analysis

PBMCs were isolated from patients with rheumatoid arthritis (treated with methotrexate) and stimulated with IgG (blue bars) or abatacept (red bars) for 30 min, 1 hour, 3 hours, or 12 hours. Gene expression was measured by quantitative real-time PCR using the following primers: *nfatc1*, 5′-GTCCTGTCTGGCCACAAC-3′ and 5′-GGTCAGTTTT-CGCTTCCATC-3′; *c-fos*, 5′-CACTCCAAGCGGAGACAGAC-3′ and 5′-AGGTCATCAGGGATCTTGCAG-3′; *rank*, 5′-TCCTCCACGG-ACAAATGCAG-3′ and 5′-CAAACCGCATCGGATTTCTCT-3′; *csfr1*, 5′-CTCTGCAGGAGCCCACACGC-3′ and 5′-AGCGGACCTGG-TACTTGGGCT-3′; β-*2-microglobulin*, 5′-GATGAGTATGCCTG-CCGTGTG-3′ and 5′-CAATCCAAATGCGGCATCT-3′.

Statistical analysis

All statistical analyses were performed with Student's *t* test or one-way analysis of variance (ANOVA) followed by Tukey's test and are represented as means ± SEM unless otherwise stated. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

SUPPLEMENTARY MATERIALS

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Fig. S1. No difference in osteoblast-related bone parameters between wild-type mice and CD80/86$^{-/-}$ mice.

Fig. S2. No difference in osteoclast-related bone parameters between wild-type mice and costimulation mutants.

Fig. S3. Effects of CTLA-4 on osteoclast precursor apoptosis.

Fig. S4. Proliferation of osteoclast precursors.

Fig. S5. Osteoclast precursor numbers in CD80/86$^{-/-}$ mice.

REFERENCES AND NOTES

1. A. Bruzzaniti, R. Baron, Molecular regulation of osteoclast activity. *Rev. Endocr. Metab. Disord.* **7**, 123–139 (2006).
2. S. L. Teitelbaum, F. P. Ross, Genetic regulation of osteoclast development and function. *Nat. Rev. Genet.* **4**, 638–649 (2003).
3. Y. Y. Kong, U. Feige, I. Sarosi, B. Bolon, A. Tafuri, S. Morony, C. Capparelli, L. Li, R. Elliott, S. McCabe, T. Wong, G. Campagnuolo, E. Moran, E. R. Bogoch, G. Van, L. T. Nguyen, P. S. Ohashi, D. L. Lacey, E. Fish, W. J. Boyle, J. M. Penninger, Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand. *Nature* **402**, 304–309 (1999).
4. D. L. Lacey, E. Timms, H. L. Tan, M. J. Kelley, C. R. Dunstan, T. Burgess, R. Elliott, A. Colombero, G. Elliott, S. Scully, H. Hsu, J. Sullivan, N. Hawkins, E. Davy, C. Capparelli, A. Eli, Y. X. Qian, S. Kaufman, I. Sarosi, V. Shalhoub, G. Senaldi, J. Guo, J. Delaney, W. J. Boyle, Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell* **93**, 165–176 (1998).
5. B. R. Wong, R. Josien, S. Y. Lee, B. Sauter, H. L. Li, R. M. Steinman, Y. Choi, TRANCE (tumor necrosis factor [TNF]-related activation-induced cytokine), a new TNF family member predominantly expressed in T cells, is a dendritic cell–specific survival factor. *J. Exp. Med.* **186**, 2075–2080 (1997).
6. K. Sato, A. Suematsu, K. Okamoto, A. Yamaguchi, Y. Morishita, Y. Kadono, S. Tanaka, T. Kodama, S. Akira, Y. Iwakura, D. J. Cua, H. Takayanagi, Th17 functions as an osteoclastogenic helper T cell subset that links T cell activation and bone destruction. *J. Exp. Med.* **203**, 2673–2682 (2006).
7. H. Takayanagi, K. Ogasawara, S. Hida, T. Chiba, S. Murata, K. Sato, A. Takaoka, T. Yokochi, H. Oda, K. Tanaka, K. Nakamura, T. Taniguchi, T-cell-mediated regulation of osteoclastogenesis by signalling cross-talk between RANKL and IFN-γ. *Nature* **408**, 600–605 (2000).
8. I. B. McInnes, G. Schett, The pathogenesis of rheumatoid arthritis. *N. Engl. J. Med.* **365**, 2205–2219 (2011).
9. U. Harre, D. Georgess, H. Bang, A. Bozec, R. Axmann, E. Ossipova, P. J. Jakobsson, W. Baum, F. Nimmerjahn, E. Szarka, G. Krumbholz, E. Neumann, R. Toes, U. Scherer, A. I. Catrina, L. Klareskog, P. Jurdic, G. Schett, Induction of osteoclastogenesis and bone loss by human autoantibodies against citrullinated vimentin. *J. Clin. Invest.* **122**, 1791–1802 (2012).
10. T. Koga, M. Inui, K. Inoue, S. Kim, A. Suematsu, E. Kobayashi, T. Iwata, H. Ohnishi, T. Matozaki, T. Kodama, T. Taniguchi, H. Takayanagi, T. Takai, Costimulatory signals mediated by the ITAM motif cooperate with RANKL for bone homeostasis. *Nature* **428**, 758–763 (2004).
11. M. Seeling, U. Hillenhoff, J. P. David, G. Schett, J. Tuckermann, A. Lux, F. Nimmerjahn, Inflammatory monocytes and Fcγ receptor IV on osteoclasts are critical for bone destruction during inflammatory arthritis in mice. *Proc. Natl. Acad. Sci. U.S.A.* **110**, 10729–10734 (2013).
12. M. Alnaeeli, J. Park, D. Mahamed, J. M. Penninger, Y. T. Teng, Dendritic cells at the osteo-immune interface: Implications for inflammation-induced bone loss. *J. Bone Miner. Res.* **22**, 775–780 (2007).
13. A. Wakkach, A. Mansour, R. Dacquin, E. Coste, P. Jurdic, G. F. Carle, C. Blin-Wakkach, Bone marrow microenvironment controls the in vivo differentiation of murine dendritic cells into osteoclasts. *Blood* **112**, 5074–5083 (2008).
14. F. Borriello, M. P. Sethna, S. D. Boyd, A. N. Schweitzer, E. A. Tivol, D. Jacoby, T. B. Strom, E. M. Simpson, G. J. Freeman, A. H. Sharpe, B7-1 and B7-2 have overlapping, critical roles in immunoglobulin class switching and germinal center formation. *Immunity* **6**, 303–313 (1997).
15. A. Shahinian, K. Pfeffer, K. P. Lee, T. M. Kündig, K. Kishihara, A. Wakeham, K. Kawai, P. S. Ohashi, C. B. Thompson, T. W. Mak, Differential T cell costimulatory requirements in CD28-deficient mice. *Science* **261**, 609–612 (1993).
16. T. L. Walunas, C. Y. Bakker, J. A. Bluestone, CTLA-4 ligation blocks CD28-dependent T cell activation. *J. Exp. Med.* **183**, 2541–2550 (1996).
17. O. Manches, M. V. Fernandez, J. Plumas, L. Chaperot, N. Bhardwaj, Activation of the non-canonical NF-κB pathway by HIV controls a dendritic cell immunoregulatory phenotype. *Proc. Natl. Acad. Sci. U.S.A.* **109**, 14122–14127 (2012).
18. S. Ferrari-Lacraz, M. Sebbag, R. Chicheportiche, C. Foulquier, G. Serre, J. M. Dayer, Contact with stimulated T cells up-regulates expression of peptidylarginine deiminase 2 and 4 by human monocytes. *Eur. Cytokine Netw.* **23**, 36–44 (2012).
19. R. Josien, B. R. Wong, H. L. Li, R. M. Steinman, Y. Choi, TRANCE, a TNF family member, is differentially expressed on T cell subsets and induces cytokine production in dendritic cells. *J. Immunol.* **162**, 2562–2568 (1999).
20. A. Boasso, J. P. Herbeuval, A. W. Hardy, C. Winkler, G. M. Shearer, Regulation of indoleamine 2,3-dioxygenase and tryptophanyl-tRNA-synthetase by CTLA-4-Fc in human CD4$^+$ T cells. *Blood* **105**, 1574–1581 (2005).
21. F. Fallarino, U. Grohmann, C. Vacca, R. Bianchi, C. Orabona, A. Spreca, M. C. Fioretti, P. Puccetti, T cell apoptosis by tryptophan catabolism. *Cell Death Differ.* **9**, 1069–1077 (2002).
22. A. L. Mellor, D. H. Munn, IDO expression by dendritic cells: Tolerance and tryptophan catabolism. *Nat. Rev. Immunol.* **4**, 762–774 (2004).
23. D. H. Munn, M. D. Sharma, J. R. Lee, K. G. Jhaver, T. S. Johnson, D. B. Keskin, B. Marshall, P. Chandler, S. J. Antonia, R. Burgess, C. L. Slingluff Jr., A. L. Mellor, Potential regulatory function

of human dendritic cells expressing indoleamine 2,3-dioxygenase. *Science* **297**, 1867–1870 (2002).
24. M. C. Genovese, J. C. Becker, M. Schiff, M. Luggen, Y. Sherrer, J. Kremer, C. Birbara, J. Box, K. Natarajan, I. Nuamah, T. Li, R. Aranda, D. T. Hagerty, M. Dougados, Abatacept for rheumatoid arthritis refractory to tumor necrosis factor α inhibition. *N. Engl. J. Med.* **353**, 1114–1123 (2005).
25. F. S. Hodi, S. J. O'Day, D. F. McDermott, R. W. Weber, J. A. Sosman, J. B. Haanen, R. Gonzalez, C. Robert, D. Schadendorf, J. C. Hassel, W. Akerley, A. J. van den Eertwegh, J. Lutzky, P. Lorigan, J. M. Vaubel, G. P. Linette, D. Hogg, C. H. Ottensmeier, C. Lebbé, C. Peschel, I. Quirt, J. I. Clark, J. D. Wolchok, J. S. Weber, J. Tian, M. J. Yellin, G. M. Nichol, A. Hoos, W. J. Urba, Improved survival with ipilimumab in patients with metastatic melanoma. *N. Engl. J. Med.* **363**, 711–723 (2010).
26. H. Li, S. Hong, J. Qian, Y. Zheng, J. Yang, Q. Yi, Cross talk between the bone and immune systems: Osteoclasts function as antigen-presenting cells and activate $CD4^+$ and $CD8^+$ T cells. *Blood* **116**, 210–217 (2010).
27. H. Kelchtermans, L. Geboes, T. Mitera, D. Huskens, G. Leclercq, P. Matthys, Activated $CD4^+CD25^+$ regulatory T cells inhibit osteoclastogenesis and collagen-induced arthritis. *Ann. Rheum. Dis.* **68**, 744–750 (2009).
28. Y. G. Kim, C. K. Lee, S. S. Nah, S. H. Mun, B. Yoo, H. B. Moon, Human CD4+CD25+ regulatory T cells inhibit the differentiation of osteoclasts from peripheral blood mononuclear cells. *Biochem. Biophys. Res. Commun.* **357**, 1046–1052 (2007).
29. M. M. Zaiss, R. Axmann, J. Zwerina, K. Polzer, E. Gückel, A. Skapenko, H. Schulze-Koops, N. Horwood, A. Cope, G. Schett, Treg cells suppress osteoclast formation: A new link between the immune system and bone. *Arthritis Rheum.* **56**, 4104–4112 (2007).
30. M. M. Zaiss, K. Sarter, A. Hess, K. Engelke, C. Böhm, F. Nimmerjahn, R. Voll, G. Schett, J. P. David, Increased bone density and resistance to ovariectomy-induced bone loss in FoxP3-transgenic mice based on impaired osteoclast differentiation. *Arthritis Rheum.* **62**, 2328–2338 (2010).
31. R. Axmann, S. Herman, M. Zaiss, S. Franz, K. Polzer, J. Zwerina, M. Herrmann, J. Smolen, G. Schett, CTLA-4 directly inhibits osteoclast formation. *Ann. Rheum. Dis.* **67**, 1603–1609 (2008).
32. P. Feunou, S. Vanwetswinkel, F. Gaudray, M. Goldman, P. Matthys, M. Y. Braun, $Foxp3^+CD25^+$ T regulatory cells stimulate IFN-γ-independent CD152-mediated activation of tryptophan catabolism that provides dendritic cells with immune regulatory activity in mice unresponsive to staphylococcal enterotoxin B. *J. Immunol.* **179**, 910–917 (2007).
33. T. Onodera, M. H. Jang, Z. Guo, M. Yamasaki, T. Hirata, Z. Bai, N. M. Tsuji, D. Nagakubo, O. Yoshie, S. Sakaguchi, Constitutive expression of IDO by dendritic cells of mesenteric lymph nodes: Functional involvement of the CTLA-4/B7 and CCL22/CCR4 interactions. *J. Immunol.* **183**, 5608–5614 (2009).
34. C. Orabona, M. T. Pallotta, C. Volpi, F. Fallarino, C. Vacca, R. Bianchi, M. L. Belladonna, M. C. Fioretti, U. Grohmann, P. Puccetti, O. Takikawa, M. Miyasaka, SOCS3 drives proteasomal degradation of indoleamine 2,3-dioxygenase (IDO) and antagonizes IDO-dependent tolerogenesis. *Proc. Natl. Acad. Sci. U.S.A.* **105**, 20828–20833 (2008).
35. D. Von Bubnoff, M. Scheler, H. Wilms, R. Fimmers, T. Bieber, Identification of IDO-positive and IDO-negative human dendritic cells after activation by various proinflammatory stimuli. *J. Immunol.* **186**, 6701–6709 (2011).
36. D. V. Novack, L. Yin, A. Hagen-Stapleton, R. D. Schreiber, D. V. Goeddel, F. P. Ross, S. L. Teitelbaum, The IκB function of NF-κB2 p100 controls stimulated osteoclastogenesis. *J. Exp. Med.* **198**, 771–781 (2003).
37. S. Herman, R. B. Müller, G. Krönke, J. Zwerina, K. Redlich, A. J. Hueber, H. Gelse, E. Neumann, U. Müller-Ladner, G. Schett, Induction of osteoclast-associated receptor, a key osteoclast costimulation molecule, in rheumatoid arthritis. *Arthritis Rheum.* **58**, 3041–3050 (2008).
38. P. Li, E. M. Schwarz, R. J. O'Keefe, L. Ma, R. J. Looney, C. T. Ritchlin, B. F. Boyce, L. Xing, Systemic tumor necrosis factor α mediates an increase in peripheral $CD11b^{high}$ osteoclast precursors in tumor necrosis factor α–transgenic mice. *Arthritis Rheum.* **50**, 265–276 (2004).
39. F. Grassi, G. Tell, M. Robbie-Ryan, Y. Gao, M. Terauchi, X. Yang, M. Romanello, D. P. Jones, M. N. Weitzmann, R. Pacifici, Oxidative stress causes bone loss in estrogen-deficient mice through enhanced bone marrow dendritic cell activation. *Proc. Natl. Acad. Sci. U.S.A.* **104**, 15087–15092 (2007).
40. M. L. Bouxsein, S. K. Boyd, B. A. Christiansen, R. E. Guldberg, K. J. Jepsen, R. Müller, Guidelines for assessment of bone microstructure in rodents using micro-computed tomography. *J. Bone Miner. Res.* **25**, 1468–1486 (2010).

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