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CTLA-4Ig (abatacept) balances bone anabolic-effects of T cells and Wnt-10b with anti-anabolic effects of osteoblastic sclerostin

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Abstract

Activated lymphocytes promote inflammation and bone destruction in rheumatoid arthritis, making T cells and B-cells therapeutic targets. Indeed, pharmacological blockade of CD28-costimulation using CTLA-4Ig (abatacept), approved for amelioration of rheumatoid arthritis, renders T cells dormant (anergic). CTLA-4Ig also promotes bone accretion in healthy mice; surprisingly, however, this effect is driven exclusively through upregulation of bone formation, rather than anti-inflammatory effects on resorption. In the study presented here, we utilized T cell receptor β gene and Wnt-10b gene knockout mice to investigate the roles of T cells and Wnt-10b in CTLA-4Ig-induced bone anabolism. Ablation of either T cells or Wnt-10b not only abolished CTLA-4Ig-induced bone anabolism, but, paradoxically, suppressed bone formation leading to bone loss. Stalled bone formation was accompanied by bone marrow stromal cell expression of the Wnt pathway inhibitor sclerostin. Our data suggest that an immuno-skeletal pivot may promote or suppress bone formation, depending on the net outcome of CTLA-4Ig action directed independently on T cells and osteoblast-lineage cells that counters Wnt-10b-induced bone anabolism, by secretion of sclerostin. While CTLA-4Ig action is tipped in favor of bone formation under physiological conditions, pathological immunodeficiency may lead to suppressed bone formation and skeletal damage.

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Author contributions

M.N.W. designed the studies, interpreted the data and wrote the manuscript. S.R-P., T.V., D.W., M.M.H. and G.R.B. performed the research. R.P. and T.F.L. provided unique reagents necessary to undertake the studies. All authors read, provided intellectual input and approved the final manuscript.

Competing interests

M.N.W., the Department of Veterans Affairs, and Emory University have filed a patent for the use of Abatacept as a bone anabolic therapy. The authors declare no other competing financial interests.

Keywords

Abatacept; CTLA-4Ig; T cells; osteoblasts; Wnt-10b

Introduction

Rheumatoid arthritis (RA) is an inflammatory autoimmune disease that causes crippling disability. RA afflicts 1–2% of the population and not only causes focal joint erosions owing to locally increased bone turnover, but also drives a global negative balance in bone remodeling leading to systemic bone loss.¹ These systemic effects of RA result in the development of osteopenia and ultimately of osteoporosis and an increased propensity to fracture. Fracture can be devastating, especially in frail and aged populations, necessitating extensive rehabilitation and diminishing quality of life.^{2, 3}

Activated lymphocytes (B cells and T cells) are implicated in the etiology of RA.⁴ However, the depth of integration between the skeletal and immune systems, the immuno-skeletal interface, has only recently begun to be appreciated.^{5, 6} Under inflammatory conditions, activated lymphocytes secrete bone active cytokines including TNF- α and the key osteoclastogenic factor Receptor activator of NF- κ B ligand (RANKL), driving up bone resorption and loss of bone mineral density (BMD) and bone mass.^{7–11}

The CD28-costimulation pharmacological inhibitor Abatacept (CTLA-4Ig) is US Food and Drug Association (FDA) approved for amelioration of inflammation in intractable cases of RA.^{12, 13} CTLA-4Ig is a synthetic pharmacological agent based on the physiological immunomodulatory receptor CTLA-4, secreted by activated T cells and regulatory T cells (T_{reg} cells). CTLA-4 calms or terminates antigen-dependent immune responses by blockading CD80 and CD86 on antigen presenting cells (APCs), the ligands for CD28. CTLA-4Ig impedes CD28-induced signaling T cells, by blocking its ligands, CD80 and CD86, on antigen presenting cells (APCs). This terminates antigen-dependent immune responses and promotes a state of T cell unresponsiveness to further antigen stimulation (anergy).¹⁴ Attesting to the anti-inflammatory and anti-resorptive activity of CTLA-4Ig, our group has reported that CTLA-4Ig blunts T cell-induced bone loss in ovariectomized mice¹⁵ and ameliorates bone loss in mice treated with continuous infusion of parathyroid hormone (PTH), a model of hyperparathyroidism, in which T cells provide permissive signals necessary for anabolic activity.¹⁶ CTLA-4Ig has also been reported to inhibit osteoclasts directly¹⁷ through the enzyme indoleamine 2,3-dioxygenase by degrading tryptophan and promoting osteoclast apoptosis.¹⁸

Although CTLA-4Ig is an anti-inflammatory factor that blocks immune activation; it has activity to suppress CTLA activity not only under pathological conditions, such as infection/inflammation, but also in physiological conditions in healthy organisms. This is because even in the absence of infection, there is constant exposure and CD80/86-dependent activation of the immune system to foreign antigens absorbed in the gut, lungs, vagina and other sites, in addition to a low-grade physiological immune renewal process involving reactions with self-antigens, that is necessary for long term maintenance of memory T cells.¹⁹

Given putative activity in physiological homeostatic immune renewal processes, we recently examined CTLA-4Ig in healthy mice and were surprised to find that CTLA-4Ig induced significant bone gain as a specific consequence of increased osteoblastic bone formation, rather than actions mediated through down-modulation of osteoclasts and bone resorption.²⁰ The mechanisms driving bone anabolism, however, remain unclear, although we reported that Wnt-10b expression was increased in bone marrow and in purified T cells from CTLA-4Ig treated mice *ex vivo*. This led us to hypothesize that by a serendipitous activity of abortive T cell activation, promoted by CTLA-4Ig-energized T cells, may secrete Wnt-10b, driving bone formation.²⁰ Indeed, we have previously reported that the bone anabolic actions of teriparatide, a fragment of human PTH, are mediated, in part, by permissive effects on T cells involving Wnt-10b-induced bone anabolism.^{21–24}

In our study here, we used mouse genetic models of Wnt-10b and T cell deficiency to demonstrate that the anabolic activity of CTLA-4Ig *in vivo* is indeed dependent on T cells and Wnt-10b production. We further identified a potential competing inhibitory action of CTLA-4Ig on bone formation through increased sclerostin expression in bone marrow, a likely consequence of osteoblast-lineage cells. Our data suggest that chronic sub-clinical inflammation is targeted by CTLA-4Ig under physiological conditions and, although low in magnitude, is sufficient to drive significant bone anabolism over a period of several months. Our data also suggest a complex immuno-skeletal pivot between stimulation and suppression of bone formation by CTLA-4Ig that is biased in favor of bone formation under physiological conditions, but may cause significant skeletal bone damage in conditions of immunodeficiency.

Materials and methods

All reagents were purchased from the Sigma-Aldrich Chemical Co. (St. Louis, MO), unless otherwise indicated.

Mice

Animal studies were approved by the Animal Care and Use Committees of the Atlanta US Department of Veterans Affairs Medical Center and of Emory University.

Mice were housed under specific pathogen free conditions and fed gamma-irradiated 5V02 mouse chow (Purina Mills, St. Louis, MO) and autoclaved water *ad libitum*.

All studies involved C57BL6 female mice. Wild-type (WT), T cell receptor beta gene (*Tcrb*) knockout (KO) mice (deficient in $\alpha\beta$ T cells; hereafter, TCR β KO) were from Jackson Labs (Bar Harbor, ME). Wnt-10b gene (*Wnt10b*) KO mice (hereafter, Wnt-10b KO) were from an Emory breeding colony and have been previously described.^{24–26}

Mice were injected intraperitoneally with 1 mg/Kg CTLA-4Ig (Orencia: Bristol-Myers Squibb) twice weekly, or with control human Ig (Lampire Biological Laboratories, Pipersville, PA) for 3 or 6 months as indicated.

Bone densitometry

Bone mineral density (BMD) in (g/cm^2) was quantified in anesthetized mice by Dual-energy X-ray absorptiometry (DEXA) using a PIXImus 2 bone densitometer (GE Medical Systems). Total body DEXA was performed and region of interest boxes placed to quantify anatomical sites including lumbar spine, femur and tibia as described.²⁷ Left and right femurs, and left and right tibias, were averaged for each mouse and means used for group calculations.

Micro-computed tomography

μ CT was performed in L3 vertebra and mid- and distal-metaphysis of the femur *ex vivo* to assess cortical and trabecular microarchitecture using a μ CT40 scanner (Scanco Medical AG, Brüttisellen, Switzerland) calibrated weekly with a factory-supplied phantom. 405 tomographic slices were taken at the L3 vertebra (2.43 mm) at a voxel size of $6\ \mu\text{m}$ (70kVp and 114 mA, and 200 ms integration time). Trabecular bone was segmented from the cortical shell beginning approximately 0.5 mm from the distal growth plate. Projection images were reconstructed using the auto-contour function for trabecular bone. Cortical bone was quantified at the femoral mid-diaphysis from 100 tomographic slices for a total of 0.6 mm. Representative vertebral samples based on mean BV/TV were reconstructed to generate 3 dimensional (3D) visual representations. Indices and units were standardized per published guidelines.²⁸

In vitro osteoblast differentiation

Mouse preosteoblastic MC3T3-E1 cells were purchased from ATCC (Manassas, VA) and differentiated to osteoblasts in α -MEM (Thermo Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA) 1% L-glutamine (Invitrogen) and 1% penicillin/streptomycin (Thermo Scientific) and $50\ \mu\text{g}$ L-ascorbate and 10 mM β -glycerophosphate. Medium was changed every 2–3 days and CTLA-4 or Ig control was added for the final 2 days of a 7-day culture.

T cell purification and Wnt-10b ELISA

T cells were purified immunomagnetically from the spleens of mice treated with Ig or CTLA4-Ig for 6 months using the EasySep Mouse T Cell Isolation Kit (Stemcell Technologies) and cultured in RPMI1640 supplemented with 10% FBS (Atlanta Biologicals) and 1% penicillin/streptomycin (Thermo Scientific) for 48 hr. Secretion of Wnt-10b was quantified by commercial ELISA (USCN Life Science).

Real time RT-PCR assays

For MC3T3 cells total RNA was extracted using TRIzol reagent (Invitrogen). Complementary DNA (cDNA) was synthesized using QuantiTech Reverse Transcription kit (Qiagen, Valencia CA). qRT-PCR was performed using VeriQuest SYBR Green qPCR master mix (Affymetrix, Santa Clara CA) on a StepOnePlus thermocycler (Applied Biosystems, Life technologies). Primers for 18s (control), Alkaline phosphatase, Osteocalcin and Sclerostin (*Sost*) were designed using qPrimerDepot software (<http://mouseprimerdepot.nci.nih.gov/>). Fold change was calculated using the $2^{(-\text{Ct})}$ method.²⁹

Primer sequences are as follows: 18S (*Rn18s*) (NR_003278): F-5'-TTGACGGAAGGGCACCACCAG-3', R-5'-GCACCACCACCCACGGAATCG-3'. Alkaline phosphatase (*Alpl*) (NM_007431): F-5'-ACAGACCCTCCCCACGAGT-3', R-5'-TGTACCTGAGATTCGTCCC-3'. Osteocalcin (*Bglap*) (NM_007541): F-5'-AAGCAGGAGGGCAATAAGGT-3', R-5'-CAAGCAGGGTTAAGCTCACA-3'. *Sost* (NM_024449.6) F-5'-ACACCACCTCCTGCCTGCCA-3', R-5'-CGCCAAACTCACCAGGGCCA-3'.

For T cells, total RNA from bone marrow and bone marrow stromal cells (BMSC) was extracted using either TRIzol reagent (Invitrogen) or an RNA isolation kit (Qiagen, Valencia, CA); real-time RT-PCR was performed on an ABI Prism 7000 (Applied Biosystems) using commercial Taqman (Applied Biosystems) Master Mix and primer sets and probes for murine *Sost* (Mm00470479), *Wnt10b* (Mm00442104) and 18S RNA Mm03928990). Fold changes were calculated using the $2^{(-Ct)}$ method²⁹ with normalization to 18S. For CD80 and CD86 we used Commercial (Qiagen, Valencia, CA) QuantiTect Primer Assay kits (Mm_Rn18s_3_SG, Mm_Cd80_1_SG and Mm_Cd86_1_SG for 18S, CD80 and CD86 respectively) and with copy normalization to 18s.

Biochemical indices of bone turnover

C-terminal telopeptide of type I collagen (CTX), a sensitive marker of *in vivo* bone resorption and osteocalcin, a sensitive marker of *in vivo* bone formation, were quantified in the serum of mice treated with CTLA-4Ig or isotype control Ig, using RATlaps (CTX) and Rat-MID (osteocalcin) ELISAs (Immunodiagnostic Systems Inc. Fountain Hills, AZ).

Statistical analysis

Statistical significance was determined using Prism V7 (GraphPad Software Inc. La Jolla, CA). Data distributions were assessed using Shapiro-Wilk normality test. Simple comparisons between 2 groups were made using unpaired, two-tailed Students *t*-test for parametric data or Mann-Whitney test for non-parametric data. Multiple group comparisons were made using one-way ANOVA with Tukey's multiple comparisons post hoc test. Prospective changes between treatment groups over time were analyzed by two-way ANOVA with Sidak's multiple comparisons post hoc test. $P < 0.05$ was considered statistically significant. Non-significant comparisons are labeled as ($p = ns$). All data are presented as mean \pm standard deviation (SD) unless otherwise indicated.

Results

We have reported that in contrast to the anti-inflammatory effects of the CD28 costimulation inhibitor CTLA-4Ig in models of disease, in healthy WT C57BL6 mice CTLA-4Ig promotes bone formation *in vivo*, however the mechanisms involved remain unclear. We previously reported that whole bone marrow and purified T cells derived from WT mice treated with CTLA-4Ig express elevated levels of Wnt-10b suggesting a putative mechanism.²⁰ To further investigate the role of T cells and of Wnt-10b in the anabolic response to CD28 neutralization *in vivo*, we injected WT, TCR β KO, and Wnt-10b KO female mice (2 months

of age and on the C57BL6 background) with either CTLA-4Ig or human IgG isotype control (Ig) antibody for 6 months.

BMD was quantified prospectively at baseline (0) and 2, 4, and 6 months of treatment using DEXA. BMD was significantly increased by CTLA-4Ig treatment in WT mice compared to Ig control at total body, lumbar spine, and femur (Figs. 1A–C, respectively), while no significant changes were observed by DEXA at the tibia (Fig. 1D). These data confirm our previous findings of BMD gain following CTLA-4Ig administration in normal WT mice.²⁰

To test a specific requirement for T cells in this anabolic response, CTLA-4Ig was administered to TCR β KO mice. No significant differences in BMD were observed at total body or at any other anatomic site (Fig. 1E–H). These data support our hypothesis that the anabolic activity of CTLA-4Ig is mediated by a mechanism that is dependent on T cells.

We next investigated a specific role of Wnt-10b in the anabolic effect of CTLA-4Ig using Wnt-10b KO mice administered CTLA-4Ig or Ig control. Wnt-10b KO mice failed to accrete BMD in response to CTLA-4Ig and, surprisingly, underwent a significant decline in BMD at total body (Fig. 1I) and at cortical rich sites including femur and tibia (Fig. 1K and 1L respectively). Decline in BMD at the lumbar spine did not reach statistical significance (Fig. 1J). Taken together, these data suggest that Wnt-10b is necessary for the anabolic effect of CTLA-4Ig.

We further analyzed microarchitectural indices of trabecular and cortical bone independently by *ex vivo* μ CT at sacrifice following 6 months of treatment with CTLA-4Ig. Trabecular bone was quantified in lumbar vertebrae and both trabecular and cortical bone in femurs.

In the vertebrae, bone volume (BV) and bone volume fraction (BV/TV), but not total volume (TV), were significantly increased in WT mice receiving CTLA-4Ig (Fig. 2A–C, respectively). The increase in BV/TV was consistent with a significant increase in structural indices including trabecular number (Tb.N) (Fig. 2D), connectivity density (Conn.D) (Fig. 2G), and volumetric bone density (TV.D) (Fig. 2H), and a decline in trabecular separation (Tb.Sp) (Fig. 2F). Trabecular thickness (Tb.Th) was unchanged (Fig. 2E).

In contrast to WT mice, BV/TV and corresponding structural indices showed no evidence of bone accretion in T-cell null TCR β KO mice or in Wnt-10b KO mice following treatment with CTLA-4Ig. In fact, in both groups there was a significant decrease in BV and BV/TV and Tb.N (Fig. 2B, C and D respectively) and in Conn.D (Fig. 2G) and TV.D (Fig. 2H) and a corresponding increase in Tb.Sp (Fig. 2F). As with WT mice, Tb.Th was unaffected by CTLA-4Ig in TCR β KO mice and in Wnt-10b KO mice (Fig. 2E).

Representative visual representations of μ CT reconstructions of vertebral trabecular bone for WT, TCR β KO, and Wnt-10b KO mice treated with either Ig or CTLA-4Ig are shown in Fig. 2I, 2J, and 2K respectively and reflect bone gain in WT and bone loss in TCR β KO mice and Wnt-10b KO mice in response to CTLA-4Ig administration.

Biochemical markers of bone formation (serum osteocalcin) and of bone resorption (serum CTx), were further quantified to evaluate bone turnover. Consistent with the anabolic

activity of CTLA-4Ig, osteocalcin was significantly increased by CTLA-4Ig treatment of WT mice, but surprisingly it was significantly suppressed in mice lacking T cells (Fig. 2L). No statistically significant changes were observed following CTLA-4Ig in mice lacking Wnt-10b, however Wnt-10b KO mice had very suppressed baseline levels of osteocalcin that may have masked differences, consistent with previous published reports.²⁵ CTx was unchanged in any group by CTLA-4Ig administration (Fig. 2M).

Trends in BV/TV and trabecular structural indices in the femoral metaphysis were similar to that observed in spine with significant gains in bone mass in WT mice treated with CTLA-4Ig, and significant declines in both TCR β KO and Wnt-10b KO mice (Fig. 3A–H).

Although cortical indices in the femur trended upwards in CTLA-4Ig treated WT mice they did not reach statistical significance in this study (Fig. 3I–L). However, cortical thickness (Ct.Th) was significantly diminished in CTLA-4Ig treated TCR β KO mice and in Wnt-10b KO mice (Fig. 3L), while total cross-sectional area inside the periosteal envelope (Tt.Ar) and cortical bone area (Ct.Ar) were also significantly diminished in CTLA-4Ig treated Wnt-10b mice (Fig. 3I and J).

Visual representations of representative μ CT reconstructions of femoral trabecular and cortical bone for WT, TCR β KO, and Wnt-10b KO mice treated with Ig or CTLA-4Ig are shown in Figure 3M, N and O respectively).

As T cells are a key target of CTLA-4Ig and known to secrete Wnt-10b, we further purified T cells (immunomagnetically) from WT mice treated with either Ig or CTLA-4Ig for 6 months and quantified Wnt-10b production in condition medium by ELISA. The data show that CTLA-4Ig leads to a significant increase in secretion of Wnt-10b (Fig. 4A).

Taken together, the data confirm an *in vivo* requirement for both T cells and Wnt-10b in the anabolic response to CTLA-4Ig, and further reveal an unexpected loss of bone mass in the absence of either T cells or Wnt-10b, resulting from suppression of bone formation.

CTLA-4Ig has been reported to mediate direct inhibitory effects on osteoclasts¹⁷ although this does not explain the potent suppression of bone formation observed in our studies. However, human³⁰ and murine³¹ osteoblasts also express CD80 and CD86 ligands and consequently could respond directly to CTLA-4Ig. One potential mechanism of CTLA-4Ig to offset T cell and Wnt-10b anabolic activity, which would moderate bone formation, is upregulation of sclerostin, a Wnt pathway antagonist and major product of osteoblast lineage cells, including osteoblasts and osteocytes. To test this possibility, we quantified expression of the sclerostin gene (*Sost*), by real time RT-PCR, in whole bone marrow isolated from WT mice treated with either Ig or CTLA-4Ig for 6 months. The data revealed a significant 9-fold increase in *Sost* expression (Fig. 4B).

Although osteocytes are considered a dominant source of sclerostin, as whole bone marrow isolation does not extract osteocytes that are entombed in the bone matrix, our data exclude osteocytes as the relevant source of elevated *Sost* mRNA in isolated bone marrow. Because osteoblasts are also a known source of sclerostin we next purified BMSC from mice treated

for 2 weeks with either Ig or CTLA-4Ig and performed RT-PCR for *Sost* expression. BMSC showed a significant increase in expression of *Sost* (Fig 4C).

For BMSC to react to CTLA-4Ig directly they would need to express CD80 and/or CD86 receptors. We thus performed RT-PCR for CD80 and CD86 transcripts and found detectable levels within the 20–30 CT range (Fig. 4D and 4E). Interestingly, *in vivo* treatment with CTLA-4Ig significantly increased expression of both CD80 and CD86 on BMSC. Whether this is an indirect effect of the global immunological milieu (BMSC also express CD40 and can directly respond to T cells²¹) or a cell autonomous positive feedback loop, is presently unknown.

To specifically investigate whether BMSC/osteoblasts respond directly to CTLA-4Ig by expressing *Sost*, we treated the murine osteoblastic cell line MC3T3-E1 with control Ig or CTLA-4Ig (1 or 10µg/ml) for the last 2 of 7 days of *in vitro* culture, under differentiating conditions and quantified *Sost* expression by real time RT-PCR. The data show a potent induction of *Sost* expression by MC3T3-E1 osteoblasts treated with CTLA-4Ig (Fig. 5A).

To test whether CTLA-4Ig further affects osteoblast differentiation *in vitro*, we also quantified expression of early (alkaline phosphatase) and late (osteocalcin) markers of osteoblast differentiation by real time RT-PCR. The data show that CTLA-4Ig had no significant effects on expression of either alkaline phosphatase (Fig. 5B) or osteocalcin (Fig. 5C), suggesting that CTLA-4Ig does not directly regulate differentiation of osteoblasts. CTLA-4Ig may however indirectly suppress osteoblastic differentiation *in vivo* by downregulation of Wnt-driven signaling through production of sclerostin, thus reducing the activity of endogenous Wnt ligands, such as Wnt-10b, as a negative feedback loop.

Discussion

On the basis of *in vitro* data we previously proposed a hypothetical model in which CTLA-4Ig promotes bone formation in healthy mice by inducing Wnt-10b in a T cell-dependent manner.²⁰ In our study here, we tested this model *in vivo*, and report that, consistent with our hypothesis, in the absence of either T cells or Wnt-10b *in vivo*, CTLA-4Ig fails to promote bone gain.

An interesting and unexpected finding in this study was that in the absence of either Wnt-10b or T cells, the net effect of CTLA-4Ig was not simply neutral, but a significant loss of bone mass. This effect was more robust in Wnt-10b KO than in TCRβ KO mice, leading to a significant decline in BMD and suppression of serum osteocalcin, a marker of *in vivo* bone formation.

BMD and bone turnover markers reflect changes across large regions of the skeleton and hence predominantly cortical effects. Given that trabecular bone is a relatively minor component of the skeleton (~20% of bone mass), the data are consistent with our µCT findings showing that Wnt-10b KO mice underwent significant loss of both cortical and trabecular bone, while TCRβ KO mice lost predominantly trabecular bone mass. Furthermore, a lack of basal Wnt10b in the Wnt-10b KO may have led to a more aggressive suppression of bone formation following CTLA4-Ig treatment, as increased sclerostin in the

face of diminished total circulating Wnt-10b may have more potently diminished bone formation, than loss of the T cell component (TCR β KO) following CTLA4-Ig treatment, given Wnt-10b from other sources such as osteoblast likely buffers basal formation in the face of increased sclerostin.

The data further suggest that T-cell-independent sources of Wnt-10b, may also contribute to CTLA-4Ig-induced bone effects. Indeed, CD4⁺ T cells provide costimulatory cues and cytokine stimulation in support of humoral immunity (B cells), cytotoxic CD8⁺ T cells, and professional APCs including macrophages, dendritic cells and B cells. Complete loss of T cells thus has a significant impact on the functioning of multiple other components of adaptive and innate immunity, some of which may contribute additional Wnt-10b to the bone microenvironment.

We have reported that osteoblasts and/or their precursors, the bone marrow stromal cells (BMSC), express the immunological costimulatory receptor CD40.³² While CD80 and CD86 ligands, the target of CTLA-4Ig, are predominately expressed on APC including monocytes (osteoclast precursors) and on mature osteoclasts themselves, they are also found on other cell types. Although CD80 and CD86 are reported to be absent on early osteoblast progenitors (mesenchymal stem cells),³³ they have been reported to be expressed on human³⁰ and mouse³¹ osteoblasts, leading us to hypothesize the existence of a direct anti-anabolic feedback loop that in the absence of T cells or Wnt-10b, leads to a net suppression of bone formation. In fact, we demonstrate here for the first time, that CTLA-4Ig promotes expression of the Wnt signaling antagonist sclerostin in whole bone marrow *in vivo*, in purified BMSC *ex vivo* and by MC3T3-E1 osteoblastic cells *in vitro*. We propose that in an intact system, CTLA-4Ig-induced Wnt-10b from T cells and other sources compensates for, and out-competes, production of sclerostin by osteoblasts. The net balance is a net increase in bone formation. By contrast, in the absence of compensation by Wnt-10b from T cells (TCR β KO and Wnt-10b KO mice) the dominant CTLA-4Ig-induced signal is osteoblastic production of sclerostin and suppression of bone formation. A hypothetical model consistent with our collective *in vitro*, *ex vivo* and *in vivo* data collected thus far is presented diagrammatically in Figure 6.

An interesting finding in our study was an increased basal BMD quantified by DEXA in the Wnt-10b KO mouse compared to WT mice, but consistent with previous Wnt-10b mouse phenotyping studies²⁵ we found significantly diminished trabecular parameters including BV, BV/TV and volumetric BMD (TV.D) in femur and vertebrae. The discrepancy between DEXA and μ CT outcomes is likely due to the fact that DEXA reflects almost exclusively cortical bone which represents 80% of bone mass. Interestingly, it has been reported that the Wnt-10b-null mouse actually has an increased bone accrual at young ages, followed by an age-dependent loss of trabecular bone³⁴. Our BMD data likely reflect this phenotypic complexity.

Typical of immunocompromised strains of mice TCR β KO mice had an elevated baseline rate of bone resorption (CTx) in our study. We have reported that this stems from a RANKL/OPG imbalance due to diminished B-cell OPG production³⁵. However, T cell deficient mice do not usually show a decline in bone formation suggesting that T cells do not

provide significant bone anabolic cues in the regulation of basal bone formation *in vivo*. The reason for this is likely a consequence of the relatively low number of antigen-activated T cells and of CTLA-4 producing anergic T cells under basal conditions. However, it is possible that following resolution of adaptive immune responses to pathological stimuli (e.g. infection), sufficient numbers of activated T cells may be rendered anergic and capable of promoting a meaningful bone anabolic response.

From a teleological perspective, a highly activated immune system is accompanied by sickness behavior preventing adequate food intake and necessitating reliance on stored energy reserves (inflammation-induced anorexia).³⁶ As bone resorption associated with immune activation liberates calcium from the skeleton to sustain biochemical functions in the body during illness,³⁶ we hypothesize that activation of bone formation may represent a necessary final step needed to replenish calcium appropriated from the skeleton during the pathological attack, once the immunological challenge has been resolved.

The net effect of physiologically produced CTLA-4 on bone formation in immunocompromised human subjects is presently unclear but we have reported that patients infected with HIV do indeed have significantly diminished serum osteocalcin, an *in vivo* marker of bone formation.³⁷ Whether this is related in part to T-cell immunodeficiency in HIV-infected subjects and the direct effects of endogenous CTLA-4 is unknown. Our findings suggest that the use of CTLA-4Ig to treat RA in immunocompromised subjects such as those with HIV-infection, even in the context of antiretroviral therapy, could have unpredictable outcomes on bone formation and should be approached with caution.

Taken together these data provide further insight into the complexity of the immuno-skeletal interface and highlight the role of the CD28 costimulatory pathway in the regulation of bone formation. Future manipulation of this pathway may be possible to promote bone accretion using pharmacological costimulation modulators such as CTLA-4Ig.

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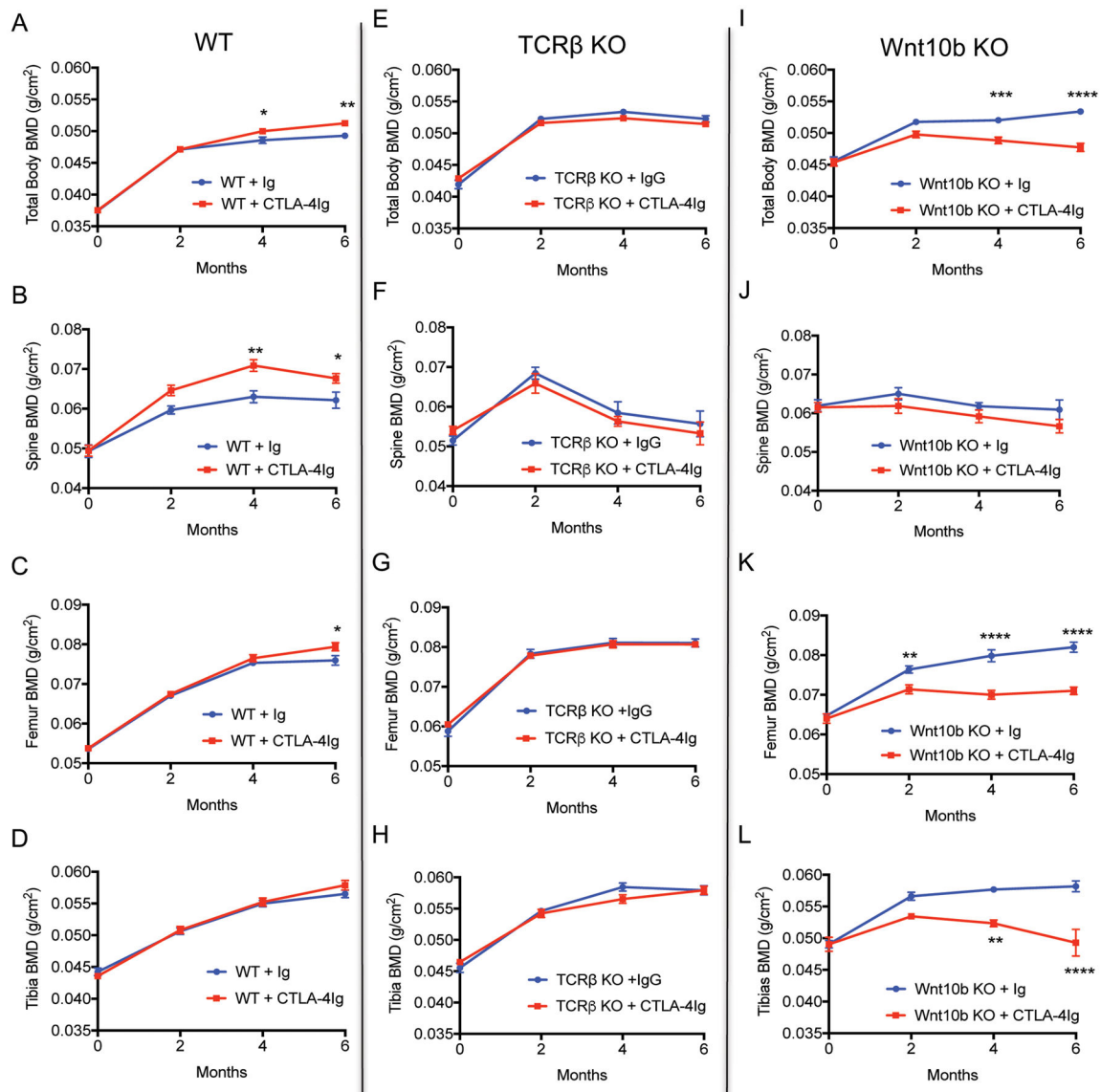


Figure 1. Effect of CTLA-4Ig on BMD of WT, TCR β KO, and Wnt-10b KO mice

BMD was quantified by DEXA in 2 months old female mice prospectively at baseline (0 time) and 2, 4 and 9 months of treatment with CTLA-4Ig or Ig control. BMD was determined in: WT C57BL6 mice at (A) total body, (B) lumbar spine, (C) femurs and (D) tibias; T-cell deficient TCR β KO mice at (E) total body, (F) lumbar spine, (G) femurs and (H) tibias and in Wnt-10b KO mice at (I) total body, (J) lumbar spine, (K) femurs and (L) tibias; $n=10-12$ mice/group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$, relative to respective Ig group by two-way ANOVA with Sidak's multiple comparisons post hoc test. All data presented as Mean \pm SD.

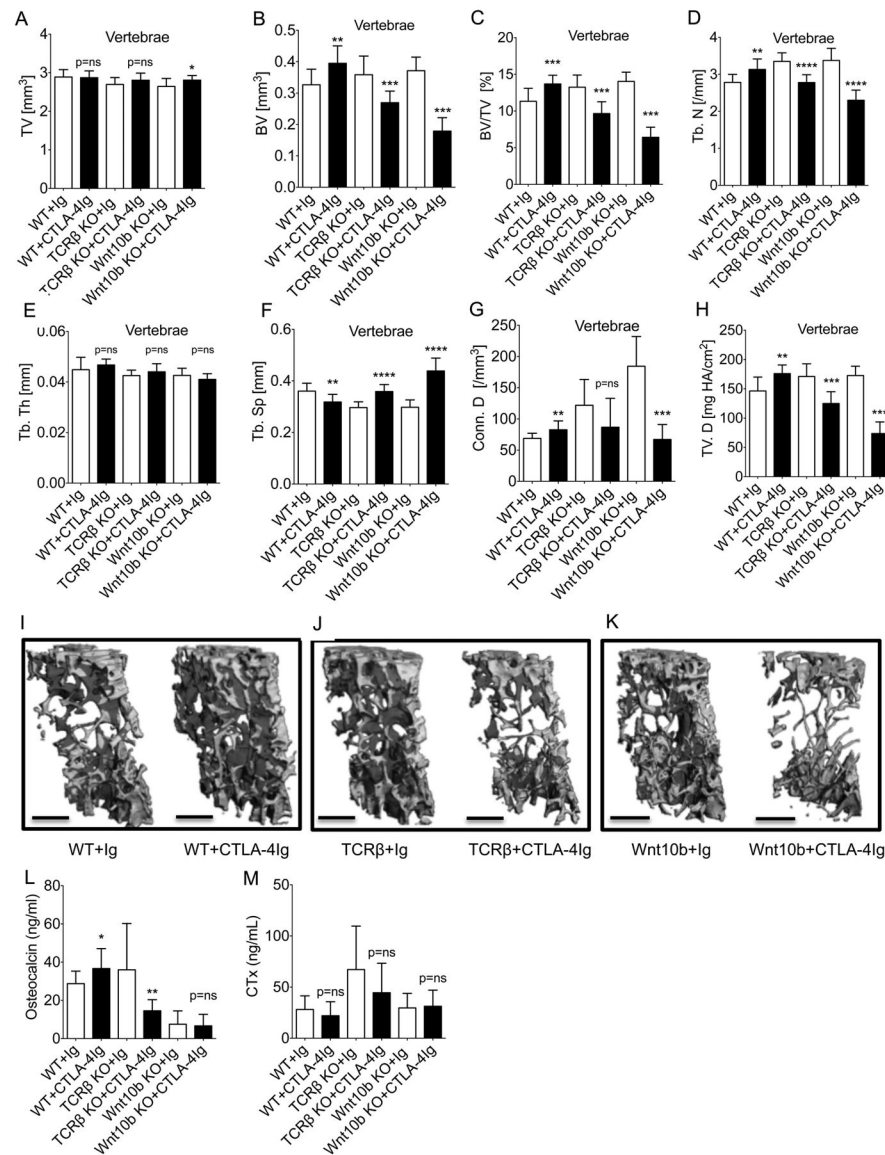


Figure 2. Vertebral μ CT analysis of WT, TCR β KO, and Wnt-10b KO mice treated with CTLA-4Ig

Trabecular vertebral μ CT indices were computed for WT, TCR β KO mice and Wnt-10b KO mice after 6 months of treatment with CTLA-4Ig or Ig control. (A) Total trabecular volume (TV), (B) trabecular bone volume (BV), (C) trabecular bone volume fraction (BV/TV), (D) trabecular number (Tb.N), (E) Trabecular thickness (Tb.Th), (F) trabecular separation (Tb.Sp), (G) connectivity density (Conn.D) and (H) volumetric density (TV.D).

Representative 6 μ m 3D reconstructions of vertebrae from WT (I), TCR β KO mice (J) and Wnt-10b KO mice (K) 6 months after CTLA-4Ig treatment. Black scale bars represent 500 μ m. Serum biochemical turnover markers for: (L) bone formation (Osteocalcin) and (M) resorption (CTx); $n = 10-12$ mice/group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ relative to respective Ig control group by Mann Whitney test. All data presented as Mean \pm SD.

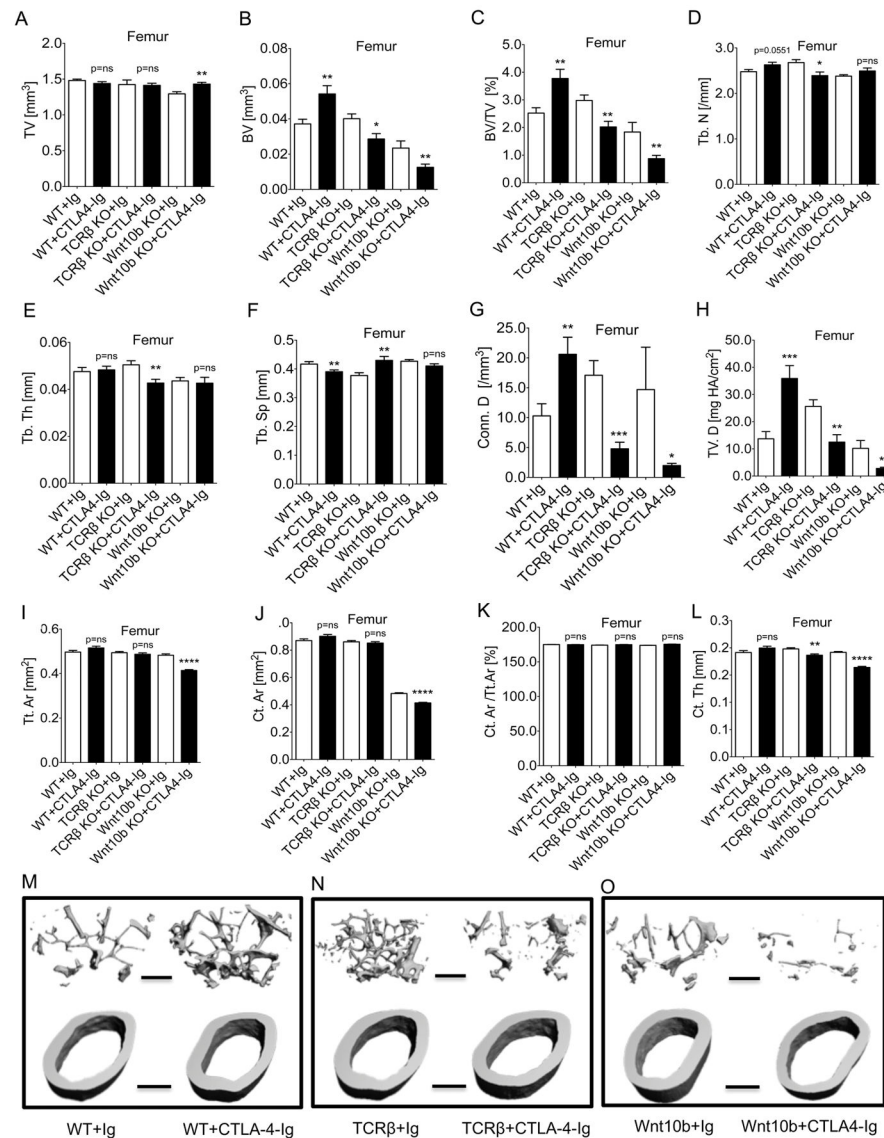


Figure 3. Femoral μ CT analysis of WT, TCR β KO, and Wnt-10b KO mice treated with CTLA-4Ig

Trabecular μ CT indices were computed in the femoral metaphases: (A) total trabecular volume (TV), (B) trabecular bone volume (BV), (C) trabecular bone volume fraction (BV/TV), (D) trabecular number (Tb.N), (E) Trabecular thickness (Tb.Th), (F) trabecular separation (Tb.Sp), (G) connectivity density (Conn.D) and (H) volumetric density (TV.D). Cortical indices were computed at femoral diaphyses for: (I) total cross-sectional area inside the periosteal envelope (Tt.Ar), (J) cortical bone area (Ct.Ar), (K) cortical area fraction (Ct.Ar/Tt.Ar) and (L) average cortical thickness (Ct.Th); $n = 10-12$ mice/group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ relative to respective Ig control group by Student's t test. All data presented as Mean \pm SD. Representative 6 μ m 3D reconstructions of trabecular and cortical compartments of femurs from WT (M), TCR β KO mice (N) and Wnt-10b KO mice (O) 6 months after CTLA-4Ig treatment. Black scale bars represent 500 μ m.

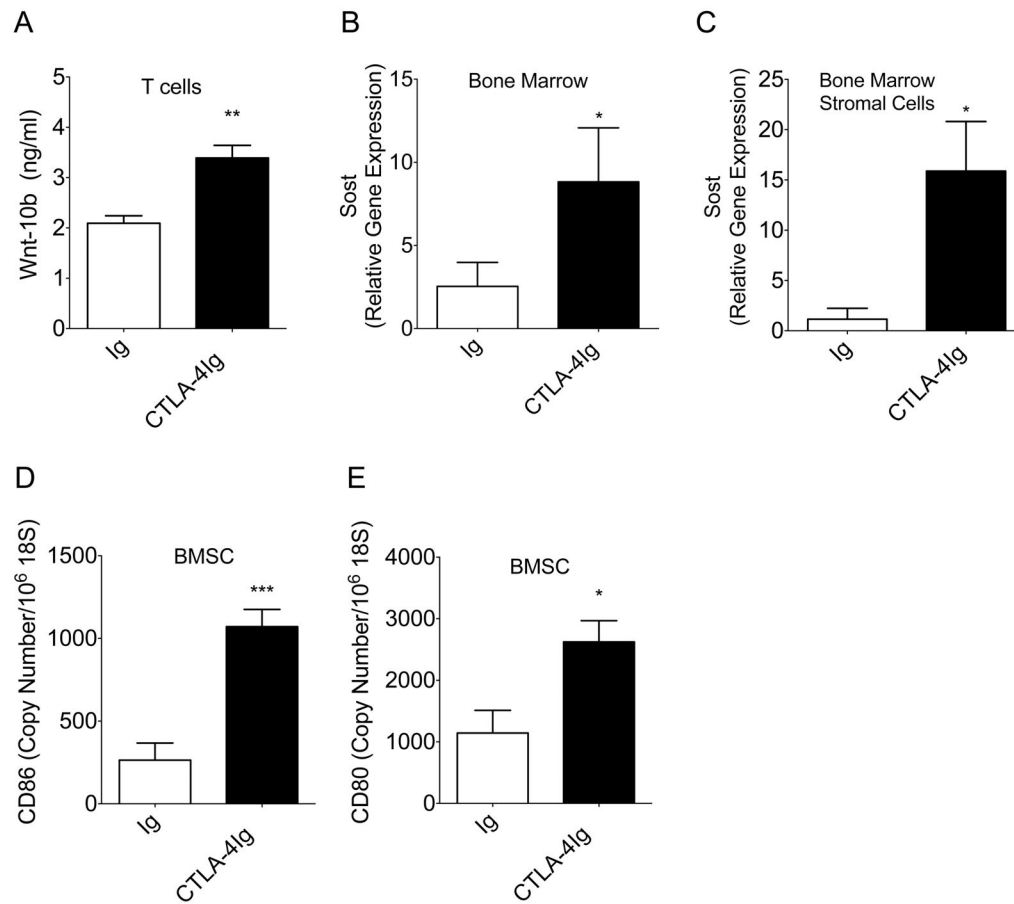


Figure 4. Effects of CTLA-4Ig on T cell Wnt10b production, bone marrow *Sost* expression and BMSC *Sost* and CD80 and CD86 expression

(A) Wnt-10b in conditioned medium (48 hr) from immunomagnetically purified T cells was quantified by ELISA and (B) RT-PCR quantification of *Sost* in whole bone marrow. WT mice were treated with Ig or CTLA-4Ig for 6 months; $n = 6$ (A) or 12 (B) mice/group. * $p < 0.05$, ** $p < 0.01$ by Mann Whitney test. (C) *Sost* expression, (D) CD86, and (E) CD80 expression, in BMSC from WT mice treated with Ig or CTLA-4Ig for 2 weeks; $n = 5$ mice/group. * $p < 0.05$ or *** $p < 0.001$ by Student t -test. Data presented as mean \pm SEM.

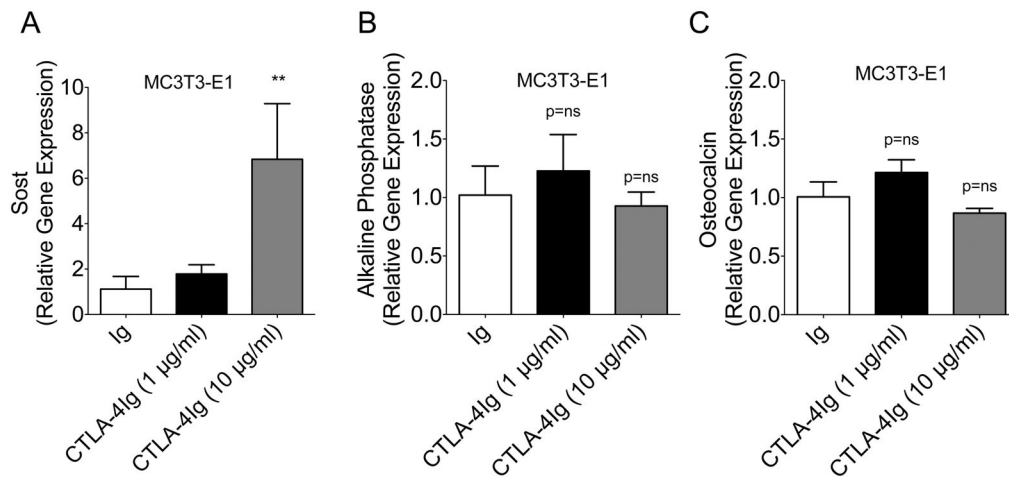


Figure 5. Effects of CTLA-4Ig on in vitro *Sost* production and osteoblast differentiation in MC3T3-E1 cells

(A) Real time RT-PCR quantification of *Sost* expression in MC3T3-E1 cells treated with CTLA-4Ig in vitro for the last 2 of 7 days. Data representative of 2 independent experiments; $n = 3$ wells/group. MC3T3-E1 osteoblast gene expression by real time RT-PCR for (B) Alkaline phosphatase (early maker of differentiation) and (C) Osteocalcin, a late marker. $N = 3$ wells/group, $**p < 0.01$ by one-way ANOVA with Tukey's multiple comparisons post hoc test. Not significant ($p = ns$). All data presented as mean \pm SD.

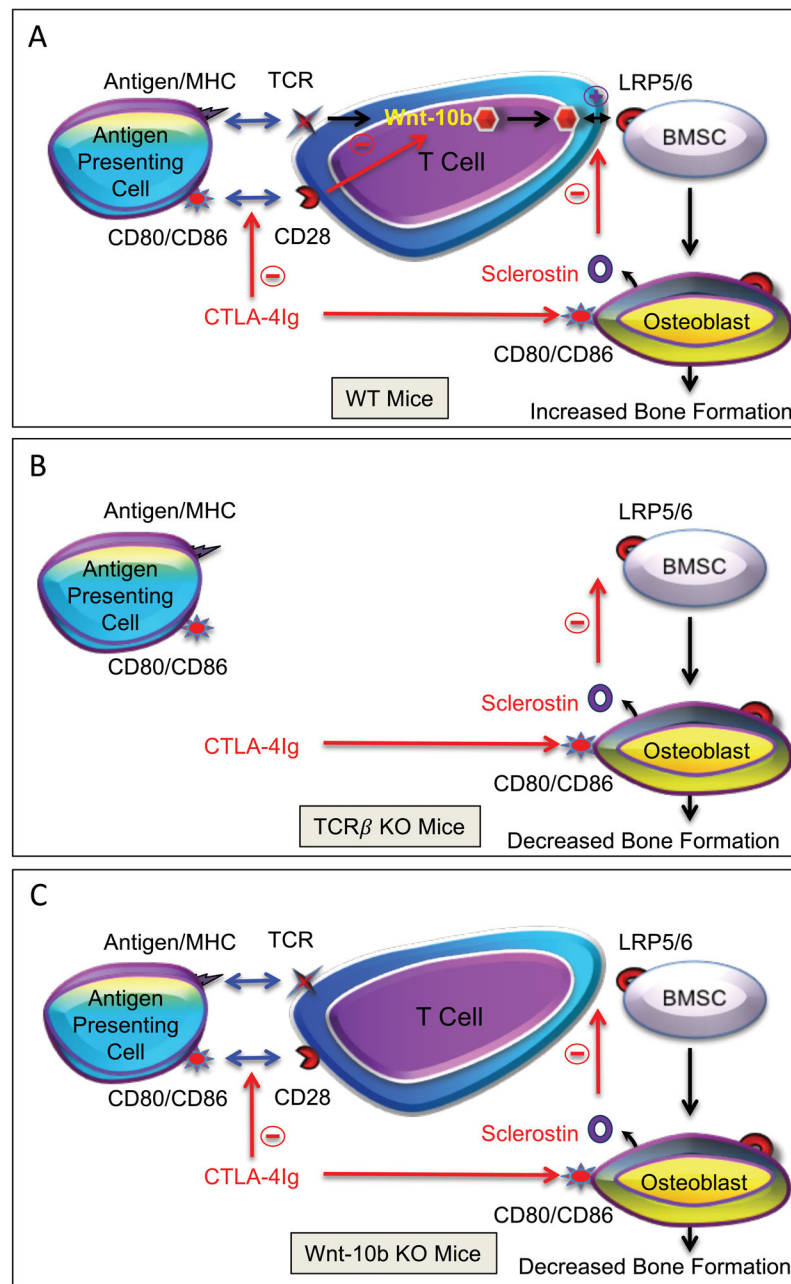


Figure 6. Hypothetical model to explain the anabolic and anti-anabolic effects of CTLA-4Ig (A) In the dual signal hypothesis of T cell activation, antigen/MHC complex expressed by antigen presenting cells binds to the T cell receptor (TCR) initiating a signal into the cell. T cell activation requires a costimulatory signal provided by T cell CD28 association with CD80/86 on the APC. Blockade of CD28 by CTLA-4Ig prevents T cell activation and induces T cell anergy with subsequent Wnt-10b production. Wnt-10b binds to low-density lipoprotein receptor-related protein 5 and 6 (5LRP5/6) receptors on bone marrow stromal cells and/or osteoblasts driving up bone formation. Bone formation is moderated by a direct negative feedback loop involving a putative CTLA-4Ig association with CD80/CD86 on the osteoblast, causing production of sclerostin, a Wnt pathway antagonist. (B) T cell deficiency

and (C) Wnt-10b deficiency, both result in loss of CTLA-4Ig-induced Wnt-10b. However, because the CTLA-4Ig-mediated sclerostin feedback loop continues to be active bone formation is further suppressed below basal levels leading to loss of bone mass.

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