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Cyclosporin A Elicits Dose-Dependent Biphasic Effects on Osteoblast Differentiation and Bone Formation

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Abstract

Cyclosporin A (CsA) is thought to prevent immune reactions after organ transplantation by inhibiting calcineurin (Cn) and its substrate, the Nuclear Factor of Activated T cells (NFAT). A dichotomy exists in describing the effects of CsA on bone formation. The concept that the suppression of Cn/NFAT signaling by CsA inhibits bone formation is not entirely supported by many clinical reports and laboratory animal studies. Gender, dosage and basal inflammatory activity have all been suggested as explanations for these seemingly contradictory reports. Here we examine the effects of varying concentrations of CsA on bone formation and osteoblast differentiation and elucidate the role of NFATc1 in this response. We show that low concentrations of CsA ($<1\mu\text{M}$ *in vitro* and 35.5 nM *in vivo*) are anabolic as they increase bone formation, osteoblast differentiation, and bone mass, while high concentrations ($>1\mu\text{M}$ *in vitro* and *in vivo*) elicit an opposite and catabolic response. The overexpression of constitutively-active NFATc1 inhibits osteoblast differentiation, and treatment with low concentrations of CsA does not ameliorate this inhibition. Treating osteoblasts with low concentrations of CsA ($<1\mu\text{M}$) increases fra-2 gene expression and protein levels in a dose-dependent manner as well as AP-1 DNA binding activity. Finally, NFATc1 silencing with siRNA increases Fra-2 expression, whereas NFATc1 overexpression inhibits Fra-2 expression. Therefore, NFATc1 negatively regulates osteoblast differentiation, and its specific inhibition may represent a viable anabolic therapy for osteoporosis.

Keywords

NFAT; osteoblasts; bone; AP-1; Fra-2; calcineurin; cyclosporin; transplantation

INTRODUCTION

Bone disease, in the form of osteopenia and osteoporosis, is a serious complication of organ transplantation in humans [1-3]. This phenomenon is not only due to the underlying disease and the organ transplantation but also is thought to be a side effect of the many drugs that are administered pre- and post- transplantation, including glucocorticoids and CsA [3-6]. However, controversial evidence exists that CsA might have a bone protective effect both in human and laboratory animals [7]. Based on the literature, both *in vivo* and *in vitro* data suggest that CsA has potential biphasic effects on bone formation and osteoblast differentiation, and

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the seemingly contradictory reports and paradoxical results stem, in part, from the wide variation in the conditions used for these studies and in the concentrations of the immunosuppressive drugs used, ranging between 1 nM-25 μ M. It has been shown that post-transplantation patients that are treated with immunosuppressive drugs including CsA develop osteoporosis [8]. Interestingly, CSA monotherapy in renal transplantation patients has been shown to significantly increase lumbar bone mineral density while transplant recipients who were treated with both CsA and glucocorticoids exhibited a significant decrease [9]. In laboratory animals, the administration of CsA to rats at high doses (15 mg/kg) shows significant bone resorption and trabecular bone loss, resulting in severe high turnover osteopenia [10]. However, administration of a lower dose of CsA (7 mg/kg) to rats results in a decrease in bone resorption and an increase in bone formation *in vivo* [11]. *In vitro*, using low concentrations of another immunosuppressive drug, FK506 (10 nM-1 μ M), has been shown to induce osteoblastic differentiation [12]. In contrast, a high concentration of FK506 (25 μ M) inhibits osteoblast differentiation [13].

The NFAT (Nuclear Factor of Activated T Cells) family of transcription factors, best known for its role in T lymphocyte activation, is composed of five proteins related to the Rel/NF κ B family (NFATc1-c4 and NFAT5) [14]. In resting cells, NFAT proteins are highly phosphorylated and reside in the cytoplasm. Upon activation by increases in intracellular Ca^{2+} , they are dephosphorylated by the Ca^{2+} /calmodulin-dependent serine-threonine phosphatase, calcineurin, (Cn); then translocate to the nucleus to become transcriptionally active [14,15]. The immunosuppressive agents CsA or FK506 are thought to elicit their response by inhibiting the Cn/NFAT signaling pathway in T cells [16]. These agents bind to cyclophilin or to FK506 binding protein, respectively. The binding proteins then bind Cn, thus inhibiting the dephosphorylation and nuclear translocation of NFAT and thus, T-cell activation [17].

The role of Cn/NFAT signaling in osteoblast differentiation and bone formation was recently reported in three different studies [13,18,19]. The findings from these reports were recently described as thought provoking for several reasons [20]. Although these studies identify osteoblast factors that could positively be regulated by Cn/NFAT signaling pathway and lead to osteoblastic anabolic response, they are inconsistent with the clinical observations that bone loss-related to CsA and Cn/NFAT inhibition is associated with high bone turnover [20,21]. It is possible that these reports are misinterpreted due to the nature of the animal models which were used in these studies [13,18,19]. These reports used global CnA alpha and NFATc1 overexpression and knockout animal models to examine the function of Cn or NFAT specifically in osteoblast differentiation and bone formation. However, these reports do not consider that Cn and NFAT are also globally expressed in different tissues and have been shown to be critical for the differentiation of many cells and organs including heart, vasculature, skeletal muscle, chondrocytes and adipocytes [22,23]. Therefore, their results may be secondary and not direct osteoblast-specific effects.

Osteoblasts are the bone forming cells. They originate from mesenchymal progenitors that, with appropriate stimulation, undergo proliferation and differentiate into preosteoblasts and then into mature, functional osteoblasts [24]. In culture, as *in vivo*, osteoblasts form bone-like mineralized nodules by undergoing three stages of development: proliferation, extracellular matrix maturation, and mineralization [25,26]. During each stage of development, specific subsets of genes are sequentially expressed or repressed. For example, histone 4 is a marker for proliferation, alkaline phosphatase for extracellular matrix maturation, and osteocalcin for mineralization [26]. Several transcription factors and signaling pathways, such as AP-1 and Runx2, have been shown to play major roles in regulating osteoblast gene expression, phenotype, and ultimately bone formation [27,28].

Activator Protein-1 (AP-1) is a dimeric transcription factor family composed of either homodimers of the Jun family (c-Jun, JunB, and JunD) or heterodimers of the Jun and Fos family (c-Fos, Fra-1, Fra-2, and FosB) [29]. Expression of the AP-1 family members varies depending on the stage of osteoblast differentiation [30]. During osteoblast proliferation, all AP-1 members are known to be expressed, while during mineralization, Fra-2 and to a lesser extent, JunD are the major AP-1 members expressed, suggesting that Fra-2 expression is critical for osteoblast differentiation and mineralization [30]. AP-1 proteins interact with other transcription factors, such as Runx2 and Smad, as well as NFAT, to regulate gene expression [31-33]. Cooperative interaction between NFAT and AP-1 on specific DNA composite sites which contain adjacent NFAT and AP-1 binding sites is essential for regulating the expression of several genes including cytokines, chemokines and cell surface receptors [34,35].

The aims of this study were to examine the potential biphasic effects of CsA on osteoblast differentiation and bone formation and to determine the role of NFATc1 in this response. Here we show paradoxical effects of CsA on bone formation and bone mass *in vivo* and osteoblast differentiation *in vitro*, resulting from different CsA concentrations. Furthermore, we demonstrate that the osteoblast specific overexpression of constitutively-active NFATc1 inhibits osteoblast differentiation. Finally, we determine the role of NFATc1 in regulating Fra-2 expression and AP-1 activation. Taken together, our work provides a critical explanation to seemingly controversial reports describing the effects of immunosuppressive agents on bone and the role of Cn/NFAT signaling on osteoblast differentiation.

MATERIALS AND METHODS

Cell Culture and Differentiation

MC3T3-E1 pre-osteoblast cells [36] were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Primary calvarial osteoblasts were isolated from 1 day-old Balb/C mice. Calvariae were subjected to three sequential digestions with collagenase (Worthington) at 37 °C. Osteoblasts were collected from the 2nd and 3rd digestions. Cells were maintained as previously described [36]. Osteoblastic induction was performed by supplementing medium with 8 mM β -glycerophosphate and 250 μ M ascorbic acid-2-phosphate [36,37].

Alkaline Phosphatase and Mineral Deposition Assays

Cultured MC3T3-E1 and primary cells were fixed in 2% v/v paraformaldehyde for 10 minutes and then incubated at 37°C with alkaline phosphatase substrate solution [36]. Mineralization was assessed by either von Kossa staining of the cultures (3 minutes in 3% w/v AgNO₃) [36] or by measurement of calcium deposition as previously described [38].

Transient Transfections and Luciferase Reporter Assays

MC3T3-E1 osteoblasts were plated at a density of 2×10^4 cells/cm² in 6-well plates. Twenty-four hours after plating, cells were transfected with 1 μ g of NFAT luciferase plasmid (Clontech) and 0.2 μ g CMV- β -galactosidase reporter construct using LipofectAMINE (Invitrogen). 24 hours post-transfection, cells were treated with CsA, as indicated. Reporter activity was measured using a luciferase (Promega) or β -galactosidase (Clontech) assay system [36,37].

Nuclear Protein Extraction and Electrophoretic Mobility Shift Assay

MC3T3-E1 Nuclear protein was extracted and the AP-1 oligonucleotide was labeled as previously described [36,38]. The oligonucleotide sequences for wild-type and mutant AP-1 were previously described [39]. Competition binding experiments were performed in the presence of a 50-fold molar excess of unlabeled wild type or mutant AP-1 oligonucleotides.

For the supershift experiments, eight μg of nuclear extracts were incubated with the ^{32}P -labeled AP-1 oligonucleotide and antibodies against Fra-2 and junD (Santa Cruz Biotechnology) overnight at 4°C .

Gene Silencing by siRNA

MC3T3-E1 Osteoblasts were plated at a density of 2×10^4 cells/ cm^2 in 6-well plates. NFATc1 or control non-functional siRNA (Santa Cruz Biotechnology) were transfected into the cells using TransIT-TKO transfection reagent as recommended by the manufacturer, (Mirus, Madison, WI). The medium was changed 24 hours post-transfection and cells were cultured in fresh medium. Cells were harvested 72 hours later [36].

Retrovirus production and infection

We used retroviral expression vectors pMSCV-GFP and pMSCV-NFATc1 [40]. Retroviruses were produced by cotransfecting pMSCV vectors with pVSV-G into BOSC23 cells using Lipofectamine (Invitrogen). Twenty four hours after transfection, the media was replaced, and retroviral supernatant was collected after 48 hours. For infection, 2×10^4 cells/ cm^2 MC3T3 cells were plated onto 6-well plates. The culture media was replaced with 500 μl of retroviral supernatant with 8 $\mu\text{g}/\text{ml}$ polybrene (Sigma) and plates were incubated for 2 hours at 37°C with 5% CO_2 . Retroviral supernatant was then removed and cells were cultured in regular growth medium.

Cyclosporin in vivo Treatment

Male, six-week old, BALB/c mice were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, Indiana). CsA was purchased from Calbiochem/EMD Biosciences (San Diego, CA) and dissolved in DMSO. CsA solution was appropriately diluted in vehicle (2% v/v DMSO, 5% v/v ethanol, 60% v/v polyethylene glycol and 33% v/v PBS) to obtain concentrations of 1, 5, 10 and 30 mg/kg body weight of the mice. Vehicle or CsA was administered daily by subcutaneous injection for 28 days. All mice were sacrificed 24 hours after the last administration of CsA. Blood samples were collected by cardiac puncture and placed into tubes containing an anticoagulant (EDTA). CsA blood levels were measured using the Abbott TdxFLx Cyclosporine Monoclonal Whole Blood assay according to manufacturer's protocol (Abbott Laboratories, Abbott Park, IL). All animal studies were approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

Dual energy X-ray absorptiometry (DXA) and μCT analysis of bone

Non-cephalic bone mineral density (BMD; g/cm^2) was determined by DXA, which was measured by a Lunar PIXImus densitometer (GE Lunar Corp, Madison, WI). After euthanizing the animals, tibiae were removed and dissected free of soft tissue. The bones were then fixed in 10% v/v formaldehyde and analyzed by a μCT system and the manufacturer's included 3-D analysis software (μCT 40, Scanco Medical, Basserdorf, Switzerland). The region of interest analyzed was the metaphysis of the proximal tibia, for a total of 25 specimens scanned. The trabecular bone in the tibia was scanned using a 12- μm slice increment on 100 slices, starting 360 μm distal to the growth plate and extending for 120 μm .

Bone histomorphometric analysis

Mouse tibiae and femora were fixed in 10% v/v buffered formalin, decalcified in EDTA, embedded in paraffin, sectioned and stained for TRAP. Also, tissues were fixed, embedded in methyl methacrylate, sectioned and stained for von Kossa. Bone histomorphometry parameters were measured using an area at least 0.5mm below growth plate, excluding the primary spongiosa and trabecular connected cortical bone [41].

Statistical analysis

All statistical analyses were performed using the Microsoft Excel data analysis program for Student's t-test analysis. Experiments were repeated at least three times unless otherwise stated. Values are expressed as the mean $\pm$ SE.

RESULTS

To resolve the contradiction regarding the effects of CsA on bone *in vivo*, we administered different concentrations of CsA (1, 5, 10 and 30 mg/kg) to mice daily by subcutaneous injections for 28 days. At the end of the study, we examined the steady-state CsA concentrations in the blood (CsA levels 24 hours after the last administration) and their effects on bone. The daily subcutaneous administration of a low dose of CsA (1 mg/kg) to mice results in a steady-state concentration of the drug of 35.5 ± 5.9 nM. The steady-state CsA concentrations averaged 1234.2 and 2230.4 nM after treatment with 10 and 30 mg/kg, respectively (Fig. 1A). Low dose CsA (1 mg/kg) dramatically increases tibial trabecular bone volume while high doses (10 and 30 mg/kg) decrease trabecular bone volume as demonstrated by representative μ CT images (Fig. 1B). Furthermore, non-cephalic whole body bone mineral density measured by DXA (Dual energy X-ray absorptiometry) is significantly increased after 28 days of treatment with low dose CsA (1 mg/kg) and decreased after treatment with high doses (10 and 30 mg/kg, respectively) (Fig. 1C). Histological analyses of femora from these animals demonstrate that treatment with low dose CsA (1 mg/kg) increases mineralization (black, von Kossa) as well as the mineral apposition rate (MAR), and decreases TRAP (Tartrate Resistant Acid Phosphatase)-positive osteoclasts (red, TRAP) compared to untreated mice (Fig. 2). In contrast, high doses of CsA (10 and 30 mg/kg) decrease trabecular bone, mineralization and MAR and increase TRAP-positive osteoclasts compared to untreated mice (Fig 2). These parameters were quantitated using BioQuant Image Analysis System[®] (R&M Biometrics, Inc, Nashville, TN) and demonstrate that the *in vivo* treatment with CsA (1 mg/kg) results in 37% increase in Bone Volume/Tissue Volume (BV/TV), a 27% increase in trabecular number and 26% decrease in trabecular separation, as compared to untreated mice. These changes in bone volume in response to a low dose of CsA (1 mg/kg) were accompanied by a significant increase in the number of osteoblasts (50%) and a 42% decrease in the number of osteoclasts. In contrast, high doses of CsA (10 and 30 mg/kg) resulted in a 20% and 36% decrease in BV/TV, 26% and 22% increase in trabecular separation, respectively. Interestingly, the decrease in bone volume is associated with a significant increase in the number of the osteoclasts (56% and 60%, respectively) without significant change in the osteoblast number (Table 1). Taken together, these results demonstrate that the *in vivo* treatment with CsA differentially affects both bone formation and resorption; ultimately leading to an increase in bone mass by low CsA concentrations and a decrease in bone mass by high CsA concentrations.

In order to directly demonstrate the effects of CsA on osteoblast differentiation, MC3T3-E1 pre-osteoblasts were used. Cells were cultured for four days in proliferation medium and osteoblastic differentiation was induced by supplementing the culture medium with β -glycerophosphate and ascorbic acid. Differentiated osteoblasts were cultured for 14 and 21 days and treated with different concentrations of CsA. At the end of the study, cells were fixed and stained for alkaline phosphatase (ALP) activity and for mineralization. Figure 3A demonstrates that low doses of CsA dose-dependently increase both ALP activity (red, upper panel) and mineralization (black, lower panel). Interestingly, although ALP and mineralization are increased in response to 1 μ M CsA compared to untreated cells, they are decreased compared to treatment with 500 nM CsA. Furthermore, calcium deposition was quantitated and shows a significant increase after treatment with 1 μ M CsA (Fig. 3B). To examine the effect of CsA on osteoblast proliferation, we performed [³H]-thymidine incorporation. Fig. 3C demonstrates that CsA treatment significantly decreases osteoblast proliferation, as measured

by [^3H]-thymidine incorporation, with a 25% decrease at 500 nM CsA and a 35% decrease at 1000 nM, while 100 nM CsA had no effect.

The increase in the osteoblastic phenotype in response to low dose CsA was confirmed by examining the expression of the early and late osteoblastic gene markers, ALP and osteocalcin, respectively. Treating osteoblasts with low concentrations of CsA (<1 μM) significantly increased *ALP* and *osteocalcin* gene expression, in a dose-dependent manner, as shown by real-time RT-PCR (Figs. 3D and E).

Consistent with numerous reports, we also demonstrate that osteoblast differentiation is inhibited when CsA is used at high concentrations (10 and 25 μM) (Fig. 4A). The decrease in differentiation was not due to an increase in apoptosis, as we show that CsA does not affect osteoblast apoptosis while treatment with 10 μM Actinomycin D for 6 hours, as a positive control [42], results a 270% increase in the number of apoptotic cells (Fig. 4B). Furthermore, we examined the dose-dependent effects of CsA on the differentiation of primary mouse calvarial osteoblasts to confirm that this response is not only limited to the MC3T3-E1 osteoblastic cell line. As expected, primary calvarial osteoblasts responded similarly to MC3T3-E1 cells after treatment with CsA (100 nM- 25 μM) for 14 and 21 days (Fig. 4C). Our results confirm that CsA elicits biphasic dose-dependent response on primary osteoblasts depending on its concentration.

In order to characterize the role of NFAT in the paradoxical effect of CsA on osteoblast differentiation we first characterized the expression of NFAT isoforms in MC3T3-E1 osteoblasts. Cells were cultured for 11 days in the presence of β -glycerophosphate and ascorbic acid. RNA, cytoplasmic (Cyto) and nuclear (Nuc) proteins were extracted and the expression of NFAT isoforms examined by RT-PCR and Western blotting. Here we show that osteoblasts express NFATc1, c2 and c3 genes (Fig. 5A). Furthermore, NFATc1 and c3 proteins were detected in osteoblasts in both the cytoplasm and in the nucleus. In contrast, NFATc2 was only detectable in the cytoplasm (Fig. 5B). Treatment with 1 μM CsA dramatically decreased the nuclear translocation of both NFATc1 and c3 (Fig. 5B). Positive controls, proteins and RNAs extracted from Jurkat cells, were examined and show expression of NFATc1, c2 and c3 (data not shown).

In order to confirm the effect of CsA on NFAT transactivation, we transfected MC3T3-E1 cells with pNFAT-TA-luciferase for 24 hours, and cells were treated with different concentrations of CsA for another 24 hours. Cells were then lysed and luciferase activity measured. Here we show that CsA significantly decreases the transactivation of NFAT at concentrations between 10 nM and 25 μM (Fig. 5C), thus demonstrating the effectiveness of CsA in inhibiting NFAT activity. Although, CsA at 1 μM concentration decreases NFAT transactivation compared to untreated osteoblasts, it slightly increases the NFAT transactivation when compared to cells treated with 500 nM CsA. The inhibition of NFATc1 nuclear translocation in osteoblasts by CsA is rapid. Three hours after treating MC3T3-E1 osteoblasts with 500 nM CsA, NFATc1 protein was mainly detected in the cytoplasm. The inhibition of NFATc1 nuclear translocation persisted even after 24 hours from the start of CsA treatment (Fig. 5D). Next, we examined NFATc1 nuclear translocation in response to different concentrations of CsA ranging between 100 nM to 25 μM . CsA at concentrations ranging from 100 nM to 25 μM inhibit the nuclear localization of active NFATc1. Interestingly, CsA when used at high concentrations (>1 μM) not only decreases nuclear NFATc1, but also the cytoplasmic inactive form of this protein (Fig. 5E). Taken together, these data suggest that the inhibition of NFAT (by low concentrations of CsA, <1 μM) induces osteoblast differentiation while high concentrations (>1 μM) inhibit it.

In order to examine the role of NFATc1 specifically in osteoblasts, we overexpressed constitutively-active NFATc1 in MC3T3-E1 cells using a retroviral system. These constructs

were a generous gift from Dr. Neil Clipstone, Northwestern University, Chicago, IL [40]. Transduction efficiency was approximately 95% (data not shown). Here we demonstrate that the overexpression of constitutively-active NFATc1 in MC3T3-E1 osteoblasts inhibits osteoblast differentiation as demonstrated by a decrease in ALP activity (red, upper panel) and mineralization (black, lower panel) (Fig. 6A). The inhibition in osteoblast differentiation was not due to a decrease in osteoblast cell number in response to an increase in NFATc1 expression. In fact, despite the decrease in differentiation, the number of the osteoblast cells that express constitutively-active NFATc1, 48 hours after culturing in serum free medium, was 25% more than that of the GFP expressing cells, as determined by MTT assay (data not shown). With the purpose of determining the role of NFATc1 in the biphasic effects of CsA on osteoblast differentiation, we treated MC3T3-E1 osteoblasts that express constitutively-active NFATc1 with different concentrations of CsA (100 nM-25 μ M) for 14 and 21 days. As expected, CsA increased the differentiation of MSCV-GFP (GFP empty vector) osteoblasts when used at low concentrations (100 and 500 nM) which is demonstrated by an increase in ALP activation and mineralization, while high CsA concentrations (10 and 25 μ M) produced the opposite results (Figs. 6B and 6C, upper panels). Interestingly, CsA at low concentrations does not rescue the inhibition of osteoblast differentiation in response to the overexpression of NFATc1 while high CsA concentration completely inhibits the basal ALP activation and mineralization (Figs. 6B and 6C, lower panels). Taken together, these results confirm that the anabolic effect of low CsA concentrations is NFATc1-dependent while the effects of the high concentrations are NFATc1-independent.

In order to elucidate the mechanism by which NFAT inhibition induces osteoblast differentiation, we examined the effects of CsA on the expression of Fra-2, an AP-1 family member. It has been shown that during the mineralization stage of differentiation, Fra-2, and to a lesser extent, JunD are the major AP-1 members in osteoblasts [30]. MC3T3-E1 cells were cultured for 11 days with different concentrations of CsA. Cells were harvested and RNA extracted. Real time RT-PCR was performed for *fra-2*. Here we show that CsA at concentrations lower than 1 μ M, dose-dependently increases the expression of *fra-2* in osteoblasts (Fig. 7A). Furthermore, CsA concentrations higher than 1 μ M failed to increase *fra-2* despite the inhibition of NFAT transactivation, which confirms the involvement of an NFAT-independent response (Fig. 7A). In addition, we examined the effects of NFAT inhibition by CsA on nuclear Fra-2 protein levels. MC3T3-E1 cells were cultured for 11 days and nuclear proteins extracted. Not surprisingly, the inhibition of NFAT by low concentrations of CsA dose-dependently increases Fra-2 nuclear protein levels while high concentrations have no effect (Fig. 7B). We also examined the nuclear protein levels of JunD, which is known to be the AP-1 partner of Fra-2 during osteoblast differentiation. Here we show that CsA does not affect JunD proteins at either low or high concentrations (Fig. 7B).

In order to demonstrate the specific role of NFATc1 in osteoblast differentiation and Fra-2 expression, we silenced NFATc1 expression using siRNA, and overexpressed constitutively-active NFATc1 in MC3T3-E1 cells. The effectiveness of the NFATc1 silencing or overexpression of NFATc1 was first confirmed by demonstrating the corresponding changes in NFATc1 protein expression, compared to osteoblasts transfected with a non-functional scrambled siRNA or transduced with GFP empty vector (Figs. 7C and 7D). NFATc1 siRNA dramatically induces Fra-2 expression compared to control siRNA without affecting JunD expression (Fig. 7C). In contrast, overexpression of a constitutively-active NFATc1 dramatically decreases Fra-2 expression compared with control MSCV-GFP infected osteoblasts without affecting JunD (Fig. 7D).

Fra-2 is a member of the Fos family of AP-1 transcription factors that form heterodimers with AP-1 Jun family members and functions as a transcriptional regulator [43]. We examined the effects of CsA at concentrations lower than 1 μ M on AP-1 DNA binding activity by performing

an electrophoretic mobility shift assay (EMSA). MC3T3-E1 osteoblasts were cultured for 11 days and treated with different concentrations of CsA. Nuclear proteins were extracted and incubated with radiolabeled oligonucleotide containing an AP-1 consensus sequence, and an EMSA was performed. Inhibition of NFAT by CsA increases AP-1 DNA binding activity dose-dependently in osteoblasts (Fig. 8A). Competition assays using excess wild-type AP-1 oligos abolish the AP-1 response while mutant oligos have no response (Fig. 8B). Furthermore, super shift analyses using antibodies to AP-1 family members confirm that Fra-2 and JunD are increased in the AP-1 DNA-protein complex (Fig. 8C). These findings suggest that inhibition of NFAT increases osteoblast differentiation which is accompanied by increased Fra-2 expression, leading to an increase in Fra-2/JunD binding to the AP-1 consensus sequence. We also examined the effects of CsA treatment on NFAT/AP-1 DNA binding activity. Consistent with literature [44,45], treatment with different concentrations of CsA effectively inhibited DNA binding activities of NFAT/AP-1 consensus sequence (data not shown). These results suggest that the activation of NFAT is critical for the activation of NFAT/AP-1 DNA binding, and AP-1 alone is not capable of substituting for NFAT on these consensus sequences.

DISCUSSION

Early reports concerning the role of Cn in regulating bone density arose from clinical observations noting that treatment with immunosuppressive drugs, CsA and FK506, is associated with increased incidence of bone fractures [46]. However, CsA monotherapy in renal transplantation patients has been reported to significantly increase lumbar bone mineral density [9]. Administration of CsA to laboratory animals also produces conflicting results ranging from increased bone formation to severe osteopenia [10,11]. It is not completely understood why CsA treatment would elicit opposing effects, but it is possible that these differences in results are due to the use of different species, genders, ages, and most importantly, different drug concentrations and duration of administration in these studies [10,11,47-49].

Reports examining the specific roles of Cn and NFAT in osteoblast differentiation are not consistent. Some studies have reported that CsA and FK506 enhance osteoblastic differentiation and bone formation both *in vivo* and *in vitro* [11-13,49], while other studies report that they decrease osteoblast differentiation by a variety of mechanisms. Consistent with the work of others, we demonstrate that osteoblast differentiation is indeed inhibited by CsA but only when used at high concentrations (10 μ M and 25 μ M). This inhibition was not due to an increase in apoptosis. Furthermore, we show that the administration of high doses of CsA (above 5 mg/kg) to mice significantly decreases bone mineral density and trabecular bone volume. In contrast, low concentrations of CsA (10 nM-1 μ M) dose-dependently increase ALP staining and mineralization. These results are consistent with studies reporting that both CsA and FK506 increase the mineralization of osteoblast-like cells [12,50]. Similarly, we observe an increase in trabecular bone volume and BMD *in vivo* after treating mice with a low dose of CsA (1 mg/kg). Our results are consistent with many reports that describe an increase in osteoblast differentiation in response to treatment with a low doses of CsA as well as those which report a decrease in bone mass in response to treatment with high doses of CsA [10, 11]. We demonstrate that these seemingly contradictory reports are potentially due to differences in the dosage of CsA. Furthermore, CsA at low concentrations increases bone mass not only by increasing bone formation but also by decreasing bone resorption as shown by the decrease in TRAP activity. The precise mechanism of this is currently not known. It is possible that treatment with a low dose of CsA acts on osteoclasts directly by inhibiting differentiation and/or activation, or indirectly by inhibiting the expression of RANKL and increasing osteoprotegerin expression by osteoblasts.

Nevertheless, dissimilarities between the *in vivo* and the *in vitro* systems exist. We found that CsA 10-500 nM is optimal for increasing ALP and mineralization of osteoblasts *in vitro*. In

contrast, the steady-state concentration of CsA in the blood that was effective in increasing bone formation and osteoblast number was 35 nM. This difference is most likely due to the *in vivo* generation of at least 6 known highly lipophilic CsA metabolites that are also effective in inhibiting the Cn/NFAT signaling pathway, which are not detected when CsA levels are measured in the blood [51]. Furthermore, high concentrations of CsA significantly decrease osteoblast proliferation *in vitro* while low concentrations have no effect. In contrast, our *in vivo* data show that low doses of CsA increase osteoblast number. This disparity is likely due to the involvement of other factors that are present *in vivo* which could act indirectly on osteoblasts, resulting in an increase in their proliferation. For example, it was shown that *in vivo* CsA treatment increases the expression of TGF-beta, which is known to induce osteoblast proliferation [52,53].

Here we demonstrate that in osteoblasts both NFATc1 and c3 proteins are cytoplasmic and nuclear while NFATc2 is only detectable in the cytoplasm. Basal Cn activity would naturally result in some nuclear localization of NFAT proteins. It is not yet known why NFATc2 is only present in the cytoplasm of osteoblasts, but it is likely due to special characteristics of this isoform [54]. Treatment with CsA significantly decreases NFAT nuclear localization and transactivation, but does not cause a complete inhibition. This could be due to the presence of several phosphorylation sites in the serine-rich gatekeeper region-2 of NFAT that are not susceptible to Cn dephosphorylation [14]. Furthermore, the inhibition of NFAT transactivation in osteoblasts by different concentrations of CsA does not appear to be linear. CsA begins to inhibit NFAT transactivation and NFAT protein nuclear localization at 10 nM and plateaus around 500 nM. Interestingly, at 1 μ M concentration, CsA decreases NFAT transactivation compared to untreated osteoblasts but increases it compared to the 500 nM-treated cells. This suggests that one of the mechanisms responsible for the paradoxical effect of CsA on osteoblasts could be a Cn and/or NFAT-independent response. It was reported that CsA decreases the activation of p38 MAP kinase [55]. Interestingly, p38 is known to phosphorylate NFAT, which leads to an increase in its nuclear export and ultimately decreases its activity [56]. Taken together, CsA in the 1 μ M range could be inhibiting both the Cn pathway leading to a decrease in NFAT transactivation as well as inhibiting p38 activation leading to a decrease in NFAT nuclear export and causing a counteracting increase in NFAT transactivation.

We examined the specific role of NFATc1 on osteoblast differentiation by overexpressing a constitutively-active NFATc1 in MC3T3-E1 cells. We show that the increase in NFATc1 expression and activation inhibits the ability of these fibroblastic pre-osteoblasts to differentiate into mature osteoblasts. Similar to our results, it was reported that the overexpression of NFATc1 in pre-adipocytes inhibits their differentiation into mature adipocytes [57]. In addition, it was shown that the increase in NFATc1 in these fibroblastic cells causes them to adopt a transformed cell type. The same phenomena could also be present in preosteoblasts. More work needs to be performed to confirm this.

Transcription factors, namely Fra-2, which is an AP-1 family member, are known to play a critical role in the differentiation of a variety of cells, including osteoblasts [30,58]. Our results demonstrate that treatment with CsA and the specific inhibition of NFATc1 by siRNA increase the expression of Fra-2. Analysis of the *fra-2* promoter [59] by a Transcription Element Search System (TESS) program [60] identified three potential NFAT consensus sequences. NFAT binding to these sites could be responsible for the negative regulation of *fra-2* expression. Nevertheless, it is possible that the increase in *fra-2* expression is indirect and results from the increase in osteoblast differentiation, after the inhibition of Cn/NFAT by CsA. Furthermore, we cannot rule out the involvement of other AP-1 family members in osteoblast differentiation in response to NFAT inhibition.

The increase in Fra-2 expression leads to a dose-dependent increase in AP-1 DNA binding activity and an increase in the formation of Fra-2/JunD heterodimers. NFAT inhibition and treatment with CsA did not change the expression of JunD, suggesting that the increase in Fra-2 is sufficient to recruit JunD and increase AP-1 DNA binding activity. It is also possible that other Jun family proteins could be interacting with Fra-2, causing the increase in AP-1 DNA binding activity. Interestingly, it has been shown previously that CsA inhibits the NFAT/AP-1 DNA binding activity [44,45]. Our data suggest that the cooperation between NFAT and AP-1 in binding to NFAT/AP-1 binding sites is not only regulated by AP-1 family members but also by NFAT. Inhibition of NFAT in osteoblasts increases AP-1 DNA binding activity. But due to the unavailability of NFAT proteins, AP-1 by itself is not able to be recruited to bind to the NFAT/AP-1 binding sites.

Finally, it was recently reported that embryonic cells from the NFATc1^{-/-} embryos exhibit a decrease in their osteoblastic potential, leading the authors to conclude that NFATc1 is critical for osteoblast differentiation [13]. This was explained by the ability of NFATc1 to enhance the transcriptional activation of osterix. These embryonic stem cells are known to be pluripotent and capable of differentiating into several lineages such as chondrocytes, myocytes, adipocytes, as well as osteoblasts [61]. Interestingly, NFAT has been shown to play a critical role in the lineage decision of stem cells to differentiate into chondrocytes, myocytes and adipocytes. Furthermore, it has been reported that the lineage decision of mesenchymal stem cells is dependent on their redox state and oxidative stress has been reported to play a role in the activation of the Cn/NFAT signaling pathway [62,63]. Therefore, the decrease in osteoblast differentiation that was observed by Koga et al [13] could possibly be due to a decrease in the lineage commitment of the NFATc1^{-/-} embryonic cells, and not a direct osteoblastic response. Similarly, it has recently been reported that the CnA Alpha^(-/-) mice display severe osteoporosis and markedly reduced mineral apposition rates [18]. Furthermore, it was also reported that animals that globally overexpress a constitutively nuclear NFATc1 variant (NFATc1^{nuc}) develop high bone mass [19]. These mice were generated by either disrupting the CnA Alpha gene, or overexpressing NFATc1 variant in embryonic cells, which were later injected into mice blastocytes [64]. These mice not only lack CnA Alpha or overexpressed NFATc1 from early stage of osteoblast differentiation before mesenchymal lineage commitment, but also had the Cn/NFAT signaling disrupted in all NFAT-dependent biological systems in the body, which could play a critical role in their skeletal phenotype. This is supported by other skeletal abnormalities that these mice exhibit, such as altered skeletal muscle phenotypes [65] and several other abnormalities that are related to the global loss of CnA Alpha or overexpression of NFATc1. This can indirectly affect bone phenotype, such as altered T-cell response or changes in muscle mass, which can ultimately and indirectly determine bone phenotype [64]. To support this argument we have generated a CnB1-osteoblast specific knockout mouse model using the Cre/lox system. Consistent with our results, we found that mice lacking the CnB1 gene in osteoblasts exhibit a significant increase in osteoblast differentiation and bone formation [66].

Our results provide new insight of a novel mechanism for osteoblast differentiation. Our work suggests that the Cn/NFAT signaling pathway plays a critical role in the negative regulation of osteoblast differentiation. NFATc1 acts, directly or indirectly, by negatively regulating *fra-2* expression. Clinically, the toxicity of CsA as a monotherapy at very low concentrations for a short period of time is not known. If CsA at the 1 mg/kg dose or other novel and non-toxic Cn inhibitors are found to be safe and without significant side effects *in vivo*, they may provide novel anabolic therapies for osteopenia and osteoporosis.

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Figure 1. CsA exhibits differential effects on bone formation *in vivo*

CsA at concentrations ranging between 1 and 30 mg/kg was subcutaneously administered to mice every day for 28 days. **(A)** Steady state levels of CsA in whole blood were measured 24 hours after the last CsA injection by fluorescence polarization immunoassay using CsA monoclonal antibody. Values were obtained from 5 mice in each group and represent the mean $\pm$ SE. **(B)** μ CT images of proximal metaphysis of tibia of CsA treated mice. The photographs shown are representative of 5 different mice in each group. **(C)** Bone mineral density was determined by using Dual-Energy X-ray absorptiometry (DXA). Values were obtained from 5 mice in each group and represent the mean $\pm$ SE of BMD of treated mice; *, $p \leq 0.03$, #, $p \leq 0.05$, **, $p \leq 0.004$ compared to those that were untreated.

Figure 2. Histological analysis of proximal femur of CsA treated animals

All mice were injected with calcein 8 days and 1 day before euthanasia. After treatment of CsA for 28 days, femora were dissected. Tissues were fixed, decalcified with EDTA, embedded in paraffin, sectioned and stained for hematoxylin and eosin staining (left column, pink) as well as tartrate-resistant acid phosphatase (TRAP) (right column, red). Also, tissues were fixed, embedded in methyl methacrylate, sectioned and stained for von Kossa (middle column, black) or examined unstained under a fluorescence microscope to visualize and measure the distance between the 2 calcein layers (white arrow) that reflects the mineral apposition rate (MAR). Representative pictures are shown from 5 different mice in each group.

Figure 3. CsA increases osteoblast differentiation *in vitro*

MC3T3-E1 cells were cultured for 14 days or 21 days and treated with different concentrations of CsA. **(A)** Cells were fixed and stained for alkaline phosphatase activity (ALP) (upper panel) or for mineralization by von Kossa (lower panel). **(B)** Then, cells were lysed and calcium content was measured. Represent the mean $\pm$ SE of the calcium content relative to protein; *, $p \leq 0.02$. **(C)** MC3T3-E1 cells were cultured for 7 days and treated with CsA. Then, cells were incubated with [³H]-thymidine and incorporation was measured. Represent the percent mean $\pm$ SE of the thymidine incorporation relative to total DNA; *, $p \leq 0.04$. After cultured 14 days, RNA was extracted and Real time RT-PCR was performed for **(D)** alkaline phosphatase, **(E)**

osteocalcin and actin. Represent the mean $\pm$ SE of ALP and osteocalcin mRNA expression relative to actin expression; *, $p \leq 0.05$ compared to control.

Figure 4. CsA exhibits differential effects on osteoblast differentiation *in vitro*

MC3T3-E1 (**A**) and primary mouse calvarial osteoblasts (**C**) were cultured and treated with either DMSO or different concentrations of CsA for 14 days (upper panel) or 21 days (lower panel). Cells were fixed and stained for ALP activity (upper panel) or for mineralization by von Kossa (lower panel). Similar results were seen in three other experiments. (**B**) MC3T3-E1 cells were cultured for 4 days and treated with CsA for 24 hours or Actinomycin D (ACT D, 10μM) for 6 hours. Cells were then harvested and stained with annexin V-FITC and PI. Apoptotic cell death was analyzed by flow cytometry. Values represent the mean $\pm$ SE of 4 separate experiments each performed in triplicate; *, $p \leq 0.05$ compared to control.

Figure 5. Osteoblasts express functional NFAT isoforms

MC3T3-E1 cells were cultured and treated with CsA (1 μ M) for 10 days. **(A)** Representative pictures of regular RT-PCR for NFATc1, c2 and c3 are shown. **(B)** Cytoplasmic and nuclear protein extracts (20 μ g/lane) were separated by SDS-PAGE. Immunoblots were developed using antibodies against NFATc1, c2, c3, actin and SP-1. **(C)** MC3T3-E1 cells were cultured and transfected with a pNFAT-TA-luciferase construct. Cells were treated with CsA and luciferase activity measured. Data are expressed relative to CMV- β -galactosidase and values represents the mean $\pm$ SE of 5 separate experiments; *, $p \leq 0.05$. **(D)** MC3T3-E1 osteoblasts were cultured for 4 days and then with 500nM of CsA for different time points as indicated. **(E)** MC3T3-E1 cells were cultured for 14 days and treated with CsA and DMSO. Cytoplasmic

and nuclear protein extracts were separated by SDS-PAGE. Immunoblots were developed using antibodies against NFATc1, actin and SP-1.

Figure 6. NFATc1 negatively regulates osteoblast differentiation

MC3T3-E1 cells were transduced with GFP-empty vector (MSCV-GFP) or the constitutively-active NFATc1 (MSCVNFATc1-GFP) retrovirus and then cultured either without (**A**) or with CsA, as indicated, for (**B**) 14 or (**C**) 21 days. Cells were fixed and stained for ALP activity (red) or for mineralization by von Kossa (black/brown). Similar results were seen in three other experiments.

Figure 7. NFATc1 negatively regulates *Fra-2* expression

MC3T3-E1 cells were cultured for 14 days and treated with different concentrations of CsA and DMSO as a control. Total RNA was extracted and real time RT-PCR was performed using primers for *fra-2*. Values were obtained from three separate experiments and represent the mean $\pm$ SE of *fra-2* mRNA expression relative to actin expression; *, $p \leq 0.01$ and # $p \leq 0.05$.

(B) Nuclear proteins were extracted and separated by SDS-PAGE. Immunoblots were developed using antibodies directed against *Fra-2* and total SP-1. (C) MC3T3-E1 cells were transfected with either control siRNA or NFATc1 siRNA. Cells were then harvested and lysed for protein extraction. (D) MC3T3-E1 cells were transduced with GFP-empty vector (MSCV-GFP) or the constitutively-active NFATc1 (MSCV-NFATc1) retrovirus and then cultured for

10 days. Immunoblots were developed using antibodies against NFATc1, Fra-2, JunD and SP-1. Shown is a representative of three separate experiments.

Figure 8. CsA increases AP-1 DNA binding activity in osteoblasts

MC3T3-E1 cells were cultured for 14 days and treated with CsA. **(A)** Nuclear protein extracts (4 μ g) were incubated with a 32 P-labeled oligonucleotide containing an AP-1 consensus sequence (5'-GCGTTGATGAGTCAGCCGAA-3') and an electrophoretic mobility shift assay was performed. Lane 1, DNA probe alone and FP is the free unbound probe. **(B)** Four micrograms of nuclear extracts from osteoblasts treated with either DMSO (C) or 1 μ M CsA were incubated with 50-fold molar excess of unlabeled AP-1 wild type (lane 3 and 4) as well as 50-fold molar excess of mutant AP-1 oligonucleotides (lane 5 and 6). The autoradiograph is representative of 2 separate experiments. **(C)** Nuclear extracts (8 μ g) were incubated with Fra-2 and JunD antibodies followed by incubation with AP-1 32 P-labeled oligonucleotide

sequence before performing an electrophoretic mobility shift assay. The autoradiograph is representative of 3 separate experiments.

Table 1

Histomorphometric quantification of femora from CsA-treated mice

CsA	Control	1mg/kg	5mg/kg	10mg/kg	30mg/kg
BV/TV	15.0 ± 0.5	20.5 ± 0.7 ^a	9.5 ± 0.3 ^b	12.0 ± 0.1	9.5 ± 0.2 ^a
Trabecular number (mm ⁻¹)	3.7 ± 0.1	4.7 ± 0.2 ^a	3.1 ± 0.3	3.1 ± 0.1 ^a	3.2 ± 0.2
Trabecular separation (µm)	230.6 ± 6.8	170.7 ± 7.5 ^a	231.8 ± 17.5	290.9 ± 14.1 ^b	283.2 ± 17.0 ^b
N.Ob/BS	5.5 ± 0.7	8.2 ± 0.3 ^b	5.4 ± 0.6	4.6 ± 1.0	4.2 ± 0.3
N.Oc/BS	1.0 ± 0.0	0.6 ± 0.1 ^a	1.4 ± 0.1 ^a	1.6 ± 0.0 ^a	1.7 ± 0.1 ^a

All values are the mean ± SEM. n=5

BV/TV : Bone Volume/Total Volume.

N.Ob/BS : Bone Number of Osteoblast/Bone Surface

N.Oc/BS : Bone Number of Osteoclast/Bone Surface

^a*P* <0.01 vs. control

^b*P* <0.05 vs. control