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The effect of TNFα secreted from macrophages activated by titanium particles on osteogenic activity regulated by WNT/BMP signaling in osteoprogenitor cells

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ABSTRACT

Wear particles are the major cause of osteolysis associated with failure of implant following total joint replacement. During this pathologic process, activated macrophages mediate inflammatory responses to increase osteoclastogenesis, leading to enhanced bone resorption. In osteolysis caused by wear particles, osteoprogenitors present along with macrophages at the implant interface may play significant roles in bone regeneration and implant osteointegration. Although the direct effects of wear particles on osteoblasts have been addressed recently, the role of activated macrophages in regulation of osteogenic activity of osteoblasts has scarcely been studied. In the present study, we examined the molecular communication between macrophages and osteoprogenitor cells that may explain the effect of wear particles on impaired bone forming activity in inflammatory bone diseases. It has been demonstrated that conditioned medium of macrophages challenged with titanium particles (Ti CM) suppresses early and late differentiation markers of osteoprogenitors, including alkaline phosphatase (ALP) activity, collagen synthesis, matrix mineralization and expression of osteocalcin and Runx2. Moreover, bone forming signals such as WNT and BMP signaling pathways were inhibited by Ti CM. Interestingly, TNFα was identified as a predominant factor in Ti CM to suppress osteogenic activity as well as WNT and BMP signaling activity. Furthermore, Ti CM or TNFα induces the expression of sclerostin (SOST) which is able to inhibit WNT and BMP signaling pathways. It was determined that over-expression of SOST suppressed ALP activity, whereas the inhibition of SOST by siRNA partially restored the effect of Ti CM on ALP activity. This study highlights the role of activated macrophages in regulation of impaired osteogenic activity seen in inflammatory conditions and provides a potential mechanism for autocrine regulation of WNT and BMP signaling mediated by TNFα via induction of SOST in osteprogenitor cells.

1. Introduction

Recent advances in biomaterials including titanium (Ti) have led to their frequent use as joint replacement and dental implants. Ti has been widely used due to its good osseointegration, excellent corrosion resistance and biocompatibility in biological fluids and high resistance/weight ratio. Unfortunately, wear debris, primarily generated from prosthetic joint articulations, modular interfaces, and non-articulating interfaces, remains the major factor limiting the survival of replaced implants by causing osteolysis around implant [1,2].

Particle phagocytosis by various cells is a critical component of biological response to implant debris [3]. Macrophages largely mediate inflammatory response in osteolytic conditions. On activation, macrophages releases matrix metalloproteinases, chemokines, and cytokines [4] including mediators of bone resorption such as TNFα and IL-1 [5]. These cellular mediators from macrophages may act in an autocrine and paracrine fashion to induce imbalance between bone formation and resorption either by enhancing the osteoclastic lineage or by acting on stromal or osteoblastic cells, leading to the loss of bone stock [6]. It has been well documented that monocyte-macrophage lineage cells exposed to diverse particles

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possess resorptive activity and can induce differentiation of osteoclast precursors [7,8]. Additionally, they may stimulate osteoblasts toward expression of the membrane-bound or soluble RANKL, as well as M-CSF further contributing to osteoclast recruitment [9]. Though bone resorption certainly contributes to the net bone loss associated with aseptic loosening of implants, impaired bone formation also appears to play an important role [10]. However, the role of osteoblasts in biomaterials related inflammation and impaired bone formation has not been elucidated completely.

Development of osteoblasts is tightly regulated by bone forming signals like WNT and BMP signaling pathways [11,12]. Activation of WNT/β-catenin signaling pathway is essential for proper bone development, and its suppression causes bone-related pathologies in humans [13]. In addition, BMPs are required for skeletal development and maintenance of adult bone homeostasis, and play an important role in bone forming process including fracture healing [14]. Futhermore, WNT and BMP signaling interact and cross-talk with each other in osteogenic cells [15]. Any alteration in these pathways in homeostatic bone milieu may affect the bone forming abilities of the osteoprogenitor cells. Recently, the induction of Wnt antagonist, DKK1, by TNFα has been linked to increased bone loss in a mouse model of inflammatory arthritis and in human Rheumatoid Arthritis [16]. Due to the critical roles of WNT and BMP signaling pathways in bone formation and their involvement in inflammatory bone diseases, their participation needs to be considered while designing a strategy for protecting bone loss in clinical setting [17].

Ti and its alloys are extensively used in different types of arthroplasty prosthesis and dental implant due to their excellent biocompatibility. On the other hand, a significant number of Ti particles have been detected in retrieval studies of tissues surrounding failed implants [18] and increased accumulation of Ti particles is expected during the course of time. To date, several studies have focused on direct effects of Ti particles on osteoblasts or macrophages [19–21]. A recent research utilized coculture of macrophages and osteoblast to study the inflammatory microenvironment showing enhanced secretion of cytokines [22], but yet no study addresses the involvement of activated macrophages in impaired osteogenesis induced by wear debris. As the interfacial membrane provides access for wear particles to enter the periprosthetic space [23] and wear particles can affect macrophages and osteoprogenitors variably, it is important to understand the functions of osteoprogenitors in response to cytokines released in their microenvironment. Therefore, in the present study we tried to evaluate the communicating mechanism between macrophages and osteoprogenator cells that may account for reduction of bone forming activity during inflammatory osteolysis caused by wear particles. To achieve this objective, conditioned medium from macrophages challenged with Ti particles was subjected to osteoprogenitors for identifying the regulatory mechanism involving canonical WNT and BMP signaling pathways.

2. Materials and methods

2.1. Preparation of Ti particles

Commercially pure Ti particles were obtained from Johnson Matthey Company (Ward Hill, MA). According to the manufacturer, histologic analysis revealed that 86% of the titanium particles were <10 μm in diameter. Ti particles were sterilized by baking at 180 °C for 6 h, followed by treatment with 70% ethanol for 48 h. Sterilized particles were suspended in phosphate-buffered saline (PBS). Only endotoxin-free particles as determined by means of Limulus assay (E-TOXATE; Sigma) were used in the present study. Ti particles were sonicated and vortexed before treatment.

2.2. Cell cultures

Murine osteoblastic cell line MC3T3-E1 (ATCC, CRL-2593) were cultured in α-minimum essential medium (α-MEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 U/ml streptomycin (Lonza, Basel, Switzerland) at 37 °C and 5% CO_2. Murine macrophage/monocyte cell line RAW 264.7 cells (ATCC, TIB-71) were maintained at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM; Sigma) containing 10% FBS, 100 U/ml penicillin, and 100 U/ml streptomycin. Human primary osteoblasts were isolated from proximal femur specimens obtained from healthy patients (58- to 80-year old) during hip replacements at orthopaedic surgery. The experimental protocol was approved by the ethics committee of Hallym University/Chuncheon Sacred Heart Hospital (Number; 2009-41, Approval date; 11-16-2009). Cancellous bone fragments were cultured as previously described [24] with minor modifications. Briefly, bone chips were minced and thoroughly rinsed with PBS prior to their sequential digestion in the presence of 1 mg/ml type I collagenase (Sigma) in serum-free MEM, at 37 °C for 1 h. The digested bone pieces were cultured in MEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 U/ml streptomycin. The characteristics of primary osteoblasts were confirmed by staining for ALP activity (Sigma) and analyzing the expression of osteogenic genes.

2.3. Collection of conditioned media

RAW 264.7 cells were plated at a density of 1.0×10^5 cells/60 mm dish in complete DMEM. After 24 h of attachment, cells were washed with PBS and stabilized with serum-free DMEM for 1 h. Then, cells were subjected to Ti particles to various ratios of cell to Ti particles (1:30, 1:60, 1:120 and 1:240). Control samples were treated with equal volume of PBS. After 3, 6, and 24 h of incubation, control (Cont CM) and Ti conditioned media (Ti CM) were collected, centrifuged to remove the cell debris if any, and stocked at 20 °C until use.

2.4. Alkaline phosphatase (ALP) activity assay

ALP activity was measured as described previously [25]. Briefly, MC3T3 E-1 cells or human primary osteoblasts were seeded at a density of 9×10^4 cells/well in 24-well plates. The cells were treated with CM or cytokines along with serum-free DMEM. After treatment for 48 h, the cells were washed twice with ice-cold PBS, and lysed with 150 μl/well of RIPA buffer. 20 μl of total cell lysate were mixed with 100 μl of CSPD substrate (Roche, Mannheim, Germany) and incubated for 30 min. The luminescence was detected on a luminometer (Glomax, Promega, Sunnyvale, CA). To normalize, the protein concentration of total cell lysate was assayed using a Protein assay kit (Bio-Rad, Hercules, CA).

2.5. Alizarin Red S and Sirius Red staining

Effect of Ti CM on collagen synthesis and mineralized matrix deposition was analyzed by Alizarin Red S and Sirius Red staining, respectively. To induce differentiation, MC3T3-E1 cells were seeded into 24-well plate and cultured in osteogenic medium (complete α-MEM with 50 μg/ml ascorbic acid, and 10 mM β-glycerolphosphate). After attachment, cells were treated with Ti CM or TNFα for 14 days and osteogenic medium was changed every second day. For Alizarin Red S staining, cells were fixed with 4% of paraformaldehyde for 1 h and stained with 40 mM Alizarin Red S solution (Sigma) at room temperature for 30 min. The stained cells were observed under microscope. To quantify mineralization, the stained cells were dissolved using 10% cetylpyridinium chloride (Sigma) for 1 h and transferred to a 96-well plate for measuring absorbance at 570 nm with spectrophotometer (SoftMax Pro 5). In case of collagen staining, cells were fixed with Bouin's fluid (Sigma) for 1 h, and stained with 1 mg/ml Sirius Red dye (Sigma) dissolved in saturated aqueous picric acid for 1 h. For quantification, the stained cells were dissolved in 0.1 N sodium hydroxide for 30 min. The optical density of the dissolved solution was measured using a spectrophotometer at 550 nm against a 0.1 N sodium hydroxide as a blank. Triplicate cultures were analyzed.

2.6. RNA isolation and real-time RT-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen). The 260/280 absorbance ratio was measured for verification of the RNA purity. The first strand cDNA was synthesized with 2 μg of total RNA using SuperScript II (Invitrogen), and one-tenth of the cDNA was used for each PCR mixture containing EXPRESS SYBR green qPCR Supermix (BioPrince, Seoul, Korea). Real-time PCR was performed using a Rotor-Gene Q (Qiagen, Hilden, Germany). The reaction was subjected to 40-cycle amplification at 95 °C for 20 s, at 60 °C for 20 s, and at 72 °C for 25 s. Relative mRNA expression of selected genes was normalized to GAPDH and quantified using the ΔΔCT method. The sequences of the PCR primers were listed in Table 1.

2.7. Luciferase reporter assay

MC3T3 E-1 cells were plated at a density of 9×10^4 cells/well in 24-well plates, and transfected using Genefectine transfection reagent (Genetrone Biotech Co., Korea) according to the manufacturer's instructions. The BRE, TopFlash, (Addgene, Cambridge, MA) or NFκB luciferase reporter (100 ng) and *Renilla* luciferase thymidine kinase construct (Invirtogen) (50 ng) were used to determine luciferase activity. Luciferase activities were measured in luminometer (Glomax, Promega, Sunnyvale,

Table 1
Primers for real-time RT-PCR.

Target	Forward primer (5′–>3′)	Reverse primer (3′–>5′)	GenBank no.
Mouse			
Osteocalcin	TGCTTGTGACGAGCTATCAG	GAGGACAGGGAGGATCAAGT	NM_007541.2
Runx2	AAGTGCGGTGCAAACTTTCT	TCTCGGTGGCTGGTAGTGA	NM_009820.4
TNFα	CATCTTCTCAAAATTCGAGTGACA	TGGGAGTAGACAAGGTACAACCC	NM_013693.2
IL-1β	AAGGGCTGCTTCCAAACCTTTGAC	ATACTGCCTGCCTGAAGCTCTTGT	NM_008361.3
IL-6	GAGGATACCACTCCCAACAGACC	AAGTGCATCATCGTTGTTCATACA	NM_031168.1
COX2	TTCAAAAGAAGTGCTGGAAAAGGT	GATCATCTCTACCTGAGTGTCTTT	NM_011198.3
DKK1	TCAGGTCCATTCTGGCCAACTCTT	TGGGCATTCCCTCCCTTCCAATAA	NM_010051.3
DKK2	ATGGCAGAATCTAGGAAGGCCACA	CGAACCCTTCTTGCGTTGTTTGGT	NM_020265.4
DKK3	AGCTGATGGAAGACACTCAGCACA	TCCTGGTGCACATGGACTGTGTTA	NM_015814.2
DKK4	ATGGTACTGGTGACCTTGCTTGGA	TCCGCGGAGCTCTTGATGTTGTTA	NM_145592.2
sFRP1	ACGAGTTGAAGTCAGAGGCCATC	ACAGTCGGCACCGTTCTTCAG	NM_013834.3
sFRP2	ATCCTGGAGACAAAGAGCAAGACC	TGACCAGATACCGGAGCGTTGATG	NM_009144.2
sFRP3	TGCAAATGTAAGCCTGTCAGAGC	TCCACAACGGCGGTCACATC	NM_011356.4
sFRP4	GTGGCGTTCAAGGATGATGCTTC	TTACTGCGACTGGTGCGACTG	NM_016687.3
sFRP5	CCCTGGACAACGACCTCTGC	CACAAAGTCACTGGAGCACATCTG	NM_018780.3
Wise	ACTGGATCGGAGGAGGCTATGG	TGTGGCTGGACTCGTTGTGC	NM_025312.3
WIF	CCACCTGAGGAGAGCTTGTACC	TGGCATTCTTTGTTGGGCTTTCC	NM_011915.2
SOST	AAAGGGAAGGGAGTGTGGAACGAA	CGCAGGCTTTACATTTGGGTGGAA	NM_024449.5
Noggin	TGAGCAAGAAGCTGAGGAGGAAGT	AGGTGCACAGACTTGGATGCCTTA	NM_008711.2
Chordin	CTTTGCTGCGCTCAAGTTTACGCT	TGTGGGATCTGTGAAACGAACCCT	NM_009893.2
Follistatin	TGGATCTTGCAACTCCATCTCGGA	TGCCCAAAGGCTATGTCAACACTG	NM_008046.2
GAPDH	TCAACAGCAACTCCCACTCTTCCA	ACCCTGTTGCTGTAGCCGTATTCA	NM_008084.2
Human			
SOST	TTCAGTGCCAAGGTCACTTCCAGA	TTCTTCCAGGAGTTTGTCAGCCGT	NM_025237.2
Osteocalcin	GCCTTTGTGTCCAAGC	GGACCCCACATCCATAG	NM_199173.4
Runx2	ACTGGGCCCTTTTTCAGA	GCGGAAGCATTCTGGAA	NM_004348.3
GAPDH	TCGACAGTCAGCCGCATCTTCTTT	ACCAAATCCGTTGACTCCGACCTT	NM_002046.3

CA), using a Dual-Luciferase Assay kit (Promega), according to the manufacturer's recommendations. Total value of reporter activity in each sample was normalized to *Renilla* luciferase activity.

2.8. Over-expression and silencing of SOST gene

For over-expression, MC3T3 E-1 cells were plated in 6-well plates at a density of 3×10^5 cells/well, and transfected with SOST/pcDNA3.1 + plasmid (1.6 μg) containing the full length of SOST or GFP/pcDNA3.1 + plasmid (Addgene) as a control using Genefectine transfection reagent. For silencing, endogenous transcription was disrupted by transfection with small interfering RNA (siRNA) for SOST (0.25 μg/well) (Santa Cruz biotechnology) using X-tremeGENE siRNA transfection reagent (Roche) according to the manufacturer's instructions. Scrambled RNA (0.25 μg/well) was used as a negative control (Santa Cruz biotechnology). After 8 h of transfection as described above, cells were treated with Ti CM for 48 h. Total cell lysate was collected and subjected to ALP activity assay and western blotting to detect the expression of SOST.

2.9. Protein isolation and Western blotting

Cells were lysed in RIPA buffer (20 mM Tris–HCl, pH 7.5, 200 mM NaCl, 1% Triton X-100, 1 mM dithiothreitol) containing protease inhibitor cocktail (Roche). The concentration of protein was measured with a Protein assay kit (BioRad) following the manufacturer's protocol. Total protein was subjected to SDS-polyacrylamide gel electrophoresis and transferred to a PVDF membrane. The blot was probed with primary antibodies; anti-IκBα (Cell Signaling Technology, Danvers, MA), anti-β-catenin (Cell Signaling Technology), anti-phosho-Smad1/5/8 (Cell Signaling Technology), anti-SOST (R&D Systems, Minneapolis, MN). As a loading control, anti-β-actin antibody (Santa Cruz biotechnology) was used. Subsequently, the blots were washed in TBST (10 mM Tris–HCl, 50 mM NaCl, 0.25% Tween 20) and incubated with a horseradish peroxidase-conjugated secondary antibody. The presence of target proteins was detected using the enhanced chemiluminescence reagents (BioNote Inc., Korea).

2.10. Neutralization of TNFα

To neutralize TNFα in Ti CM, Ti CM was pre-incubated with 10 μg/ml of anti-TNFα antibody (R&D systems) at 37 °C for 1 h, and treated to MC3T3-E1 cells for 48 h before analyzing ALP activity. As a control, Ti CM pre-incubated with non-immune isotype rat IgG (10 μg/ml) was used.

2.11. Lactate dehydrogenase (LDH) activity assay

Measurement of LDH activity was performed with cytotoxicity detection kit (Roche) according to the manufacturer's protocol as follows. Tissue culture media (10 μl) from each experimental sample was added to a 96-well plate containing 40 μl of PBS. Secondly, 50 μl of LDH reagent were added to each well and plates were incubated for 45 min at 25 °C in dark, and then enzymatic reaction was stopped by adding the stop solution (50 μl). Absorbance was read at 492 nm wavelength. Total cell lysate was served as a positive control of cell death.

2.12. MTT assay

An MTT assay was carried out to evaluate the viabilities of MC3T3 E-1 cells, and performed using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT; Sigma). Fresh MTT (5 mg/ml) was added to growing cells on 96-well plate and incubated at 37 °C for 3 h. Following the removal of supernatant, the insoluble formazan crystal was dissolved in 200 μl of dimethyl sulfoxide (DMSO) and optical density was measured at 570 nm wavelength.

2.13. ELISA

RAW 264.7 cells were incubated with Ti particles (1:120) for 3, 6, and 24 h. Ti CM was collected to determine the concentration of TNFα. Mouse TNFα ELISA kit (R&D systems) was used for a quantitative measurement according to the manufacturer's recommendations.

2.14. Statistical analysis

All the statistical data were analyzed by Graphpad Prism 5.0 (San Diego, CA) and evaluated by two-tailed Student *t* test. Value of $P < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. Suppressed osteogenic activity by Ti CM

Initially, we attempted to determine the effect of Ti CM on osteogenic activity of osteoprogenitors (MC3T3 E-1). Since induction of ALP activity is regarded as an early parameter for osteoblast differentiation, we examined the effect of Ti CM on ALP activity. Significant suppression in ALP activity was observed with 50% of collected Ti CM by using different ratio of cell to Ti particles (1:30, 1:60, 1:120 and 1:240) (Fig. 1A). Ti CM of 1:120 and 1:240 showed similar maximum suppression. To select ideal time for stimulation of macrophages with Ti particles, Ti CM collected after 3, 6 and 24 h was analyzed for suppression effect on ALP activity. Ti CM collected after 24 h showed maximum suppression in ALP activity (Fig. 1B)

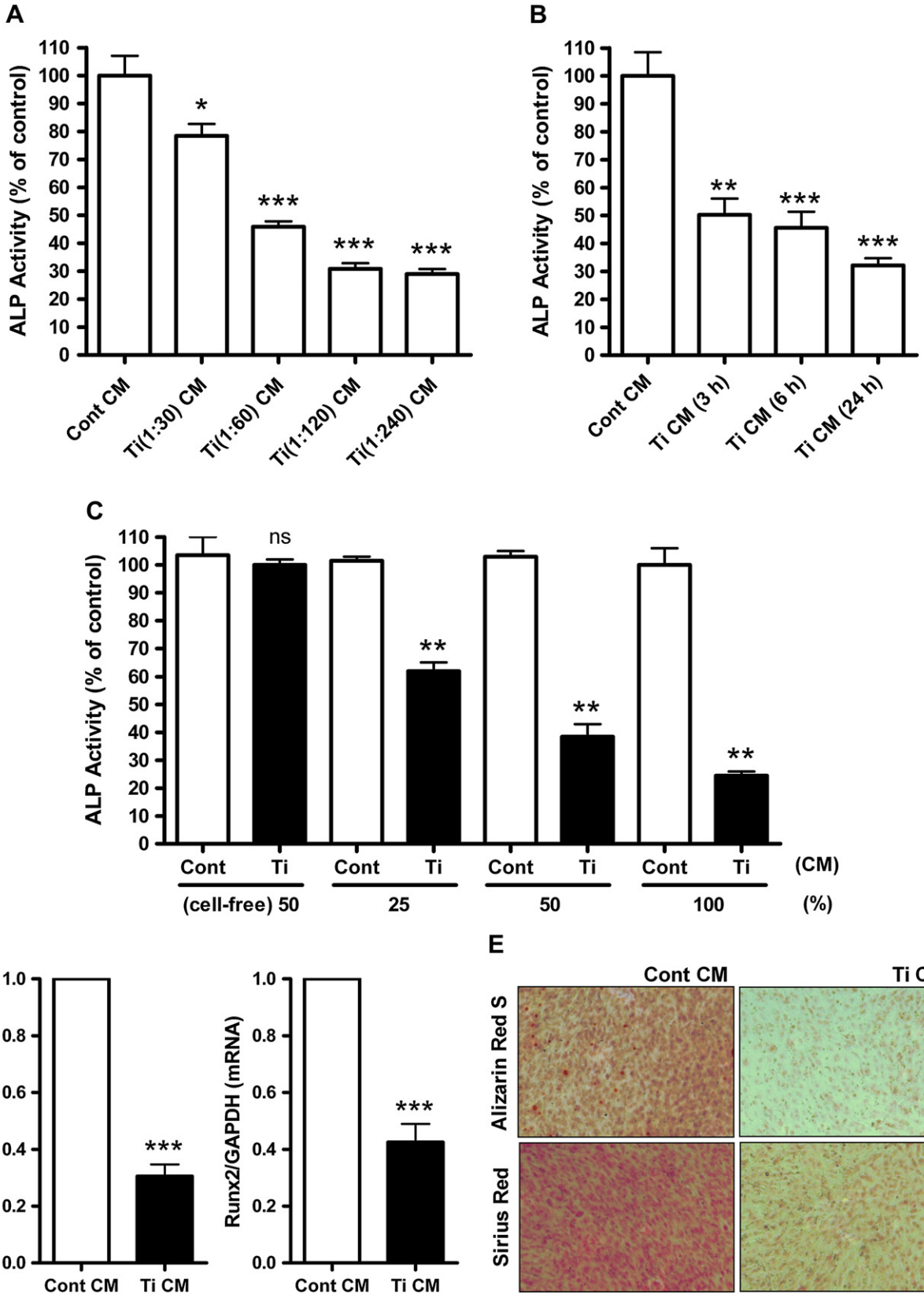

Fig. 1. Ti CM suppresses early and late differentiation markers of osteoprogenitor cells. (A) Ti CM was collected from RAW 269.4 cells treated with mentioned ratios of cell to Ti particles. (B) Ti CM was collected from RAW 269.4 cells after 3, 6, and 12 h of incubation with Ti particles (1:120). (C) MC3T3 E-1 cells were treated with various doses (25%, 50% and 100%) of Ti CM. CM collected from cell-free dish was used as a control for contamination of Ti particles. (A–C) After treatment of collected CM to MC3T3 E-1 cells for 48 h, total cell lysates were collected and analyzed for ALP activity. (D) After stimulation with Ti CM for 48 h, total cellular RNA was extracted and transcripts of osteocalcin and Runx2 were analyzed by real-time RT-PCR. Data shown are normalized to GAPDH levels. (E) MC3T3-E1 cells along with Ti CM were cultured in the osteogenic medium. Process of mineralization and collagen synthesis were visualized at day 14 by Alizarin Red S and Sirius Red staining, respectively as described in Materials and Methods. Data are shown as the mean ± SD. Similar results were obtained in three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, ***, $P < 0.001$ and ns (no significance).

with no cytotoxicity on MC3T3 E-1 cells (Supplement 1B). Therefore, Ti CM of 1:120 after 24 h was used throughout the entire study. To justify the dosage of Ti CM, suppression in ALP activity with 25%, 50% and 100% Ti CM was observed. Among these, 50% of Ti CM showed suppressive effect without any cytotoxicity and mitogenic effect (Supplement 1A & B). Also, as treatment of Ti CM collected in cell free condition showed no change in ALP activity the possibility of any Ti particle contamination in Ti CM was ruled out (Fig. 1C). In addition, the process of osteoblast differentiation is represented by upregulation of osteogenic genes such as osteocalcin and Runx2 along with induction of mineralization at terminal stages [26]. After treatment of Ti CM for 24 h, mRNA expression levels of osteocalcin and Runx2 was significantly downregulated (Fig. 1D). Furthermore, MC3T3 E-1 cells grown in osteogenic medium with treatment of Ti CM for 14 days showed suppressed Alizarin Red S and Sirius Red staining (Fig. 1E). Quantification of total mineralization and collagen synthesis by Alizarin Red S and Sirius Red extracted from stained cultures showed significant suppression in mineralization and collagen synthesis (Supplement 3A).

3.2. Suppressed activity of WNT and BMP signaling pathways by Ti CM

As WNT and BMP signaling are implicated in regulating osteoblast differentiation and bone formation [27], the effect of Ti CM on WNT and BMP signaling pathways was needed to be defined. To detect the activity of these signaling pathways, we used luciferase reporter assay. MC3T3 E-1 cells were transfected with TopFlash-luc for WNT pathway and BRE-luc for BMP pathway. After 48 h of treatment, Ti CM suppressed basal level of both TopFlash and BRE luciferase activity, demonstrating inhibitory effect of Ti CM on both pathways (Fig. 2A and B). To observe the effect of Ti CM on induced expression of WNT and BMP pathways, recombinant proteins (Wnt3a or Bmp2) were cotreated with Ti CM for 48 h. Exogenous stimulation failed to induce luciferase activity for both pathways in Ti CM treated cells, whereas induced luciferase activity was observed in Cont CM treated cells as expected. Furthermore, in order to confirm the inhibitory role of Ti CM on both signaling pathways as observed by luciferase activity, we observed the effect of exogenous stimulation on ALP activity in the presence of Ti CM. Ti CM nullified the stimulatory effect of Wnt3a and Bmp2 on ALP activity significantly as compared to Cont CM (Fig. 2C).

3.3. Identification of proinflammatory cytokines present in Ti CM

Based on the above results, we next focused on identifying the possible secreted molecules present in the Ti CM regulating osteogenic activity. Particulate debris derived from alloy implants can induce activation of monocyte-macrophage and secretion of proinflammatory cytokines like IL-1β, TNFα, and IL-6 [28]. In consistent to this report, our results of real-time RT-PCR verified that Ti particles elevate mRNA levels of TNFα, IL-1β, and IL-6 in activated macrophages, and also cause induction of COX2 (Supplement 4 & 2B). Induction of TNFα was observed to be comparatively higher (>6.0 fold) than IL-1β and IL-6 (>2.5 fold). In previous studies, TNFα and IL-1β have been shown to suppress the osteoblast differentiation [29,30]. In contrast, IL-6 produced by macrophages in response to microparticles enhances expression of osteoblast markers in MC3T3-E1 cell [31]. Therefore, to analyze the distinct role of these cytokines in Ti CM on the process of osteoblast differentiation, cells were treated with recombinant cytokines prior to ALP activity assay. Single treatment of TNFα suppressed ALP activity in a dose dependent manner, whereas various doses of IL-1β and IL-6 showed no significant change (Fig. 3A). Moreover,

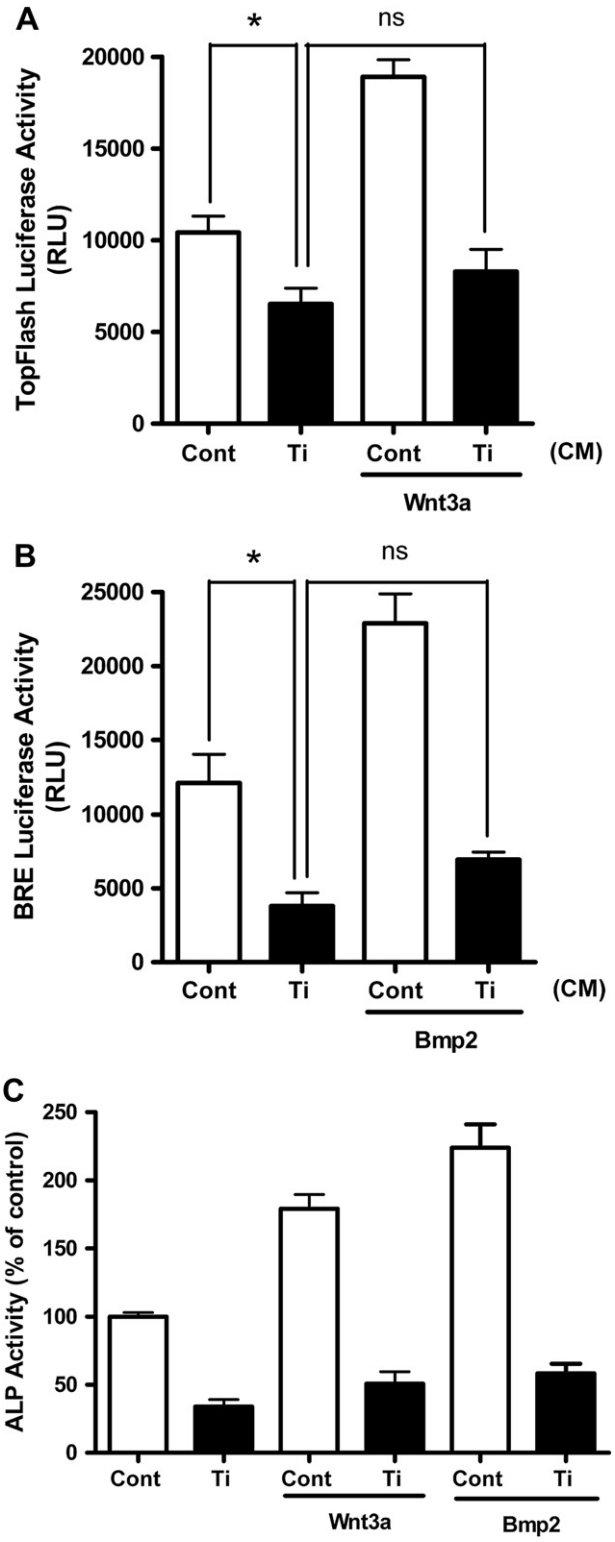

Fig. 2. Ti CM impedes WNT and BMP signaling activity at basal as well as induced level. (A & B) MC3T3 E-1 cells were transiently transfected with TopFlash-luc or BRE-luc reporter plasmids and cotreated with Ti CM and Wnt3a (50 ng/ml) or Bmp2 (100 ng/ml). After 48 h, luciferase activities were measured in cell lysates and normalized to *Renilla* luciferase activity as described in Materials and Methods. (C) Total cell lysates obtained from above samples were analyzed for ALP activity. Data are shown as the mean ± SD. Similar results were obtained in three independent experiments. *$P < 0.05$ and ns (no significance).

combinatory treatment of TNFα with IL-1β, and TNFα with IL-6 showed no significant effect, ruling out the possibility of any additive or synergistic effect of IL-1β, and IL-6 with TNFα on ALP activity (Fig. 3B). According to Gerstenfeld et al. [32], low doses of TNFα can enhance osteoblast proliferation where as high doses can inhibit bone formation *in vitro* and *in vivo*. Since the concentration of TNFα in Ti CM may be important to describe its role in regulating osteogenic activity, we quantified the amount of TNFα present in Ti CM by ELISA. The protein level of TNFα was accumulated time-dependently, and reached to a concentration of ~5 ng/ml after 24 h (Fig. 3C). As 50% of Ti CM was used for treatment, a concentration of 2 ng/ml was selected for further experiments.

3.4. Suppressed osteogenic activity by TNFα similar to Ti CM

Since TNFα alone mimicked the effect of Ti CM on ALP activity of MC3T3 E-1 cells, we further tried to analyze its possible role on WNT and BMP signaling pathways along with other osteogenic parameters. TNFα suppressed basal luciferase activity in MC3T3 E-1 cells transfected with TopFlash-luc or BRE-Luc, showing similar response to Ti CM (Fig. 4A). Furthermore, mRNA levels of both osteocalcin and Runx2 were down regulated by TNFα as depicted in Fig. 4B. Quantification of extracts from stained cultures with Alizarin Red S or Sirius Red showed significant suppression in mineralization and collagen synthesis by TNFα (Supplement 3B). To further establish whether TNFα is a predominating factor in Ti CM, neutralizing antibody was used to block the effect of TNFα. Neutralization of TNFα in Ti CM resulted in >80% recovery of ALP activity as compared to IgG control (Fig. 4C).

3.5. Involvement of NFκB activity in suppressed osteogenic activity

NFκB family of transcription factors act downstream in signal transduction mediated by TNFα [33]. Recently, it has also been documented that activation of NFκB by TNFα plays a crucial role in suppressing differentiation potential of mesenchymal stem cells to osteoblasts [34]. Consistent with these reports, TNFα was able to induce >2.5 fold of luciferase activity in MC3T3 E-1 cells transfected with NFκB-luc after 24 h of treatment. Interestingly, similar increment in luciferase activity was also detected by treatment of Ti CM (Fig. 5A). In addition, treatment of Ti CM reduced cytosolic stability of IκBα in a time-dependent manner (Fig. 5B), confirming

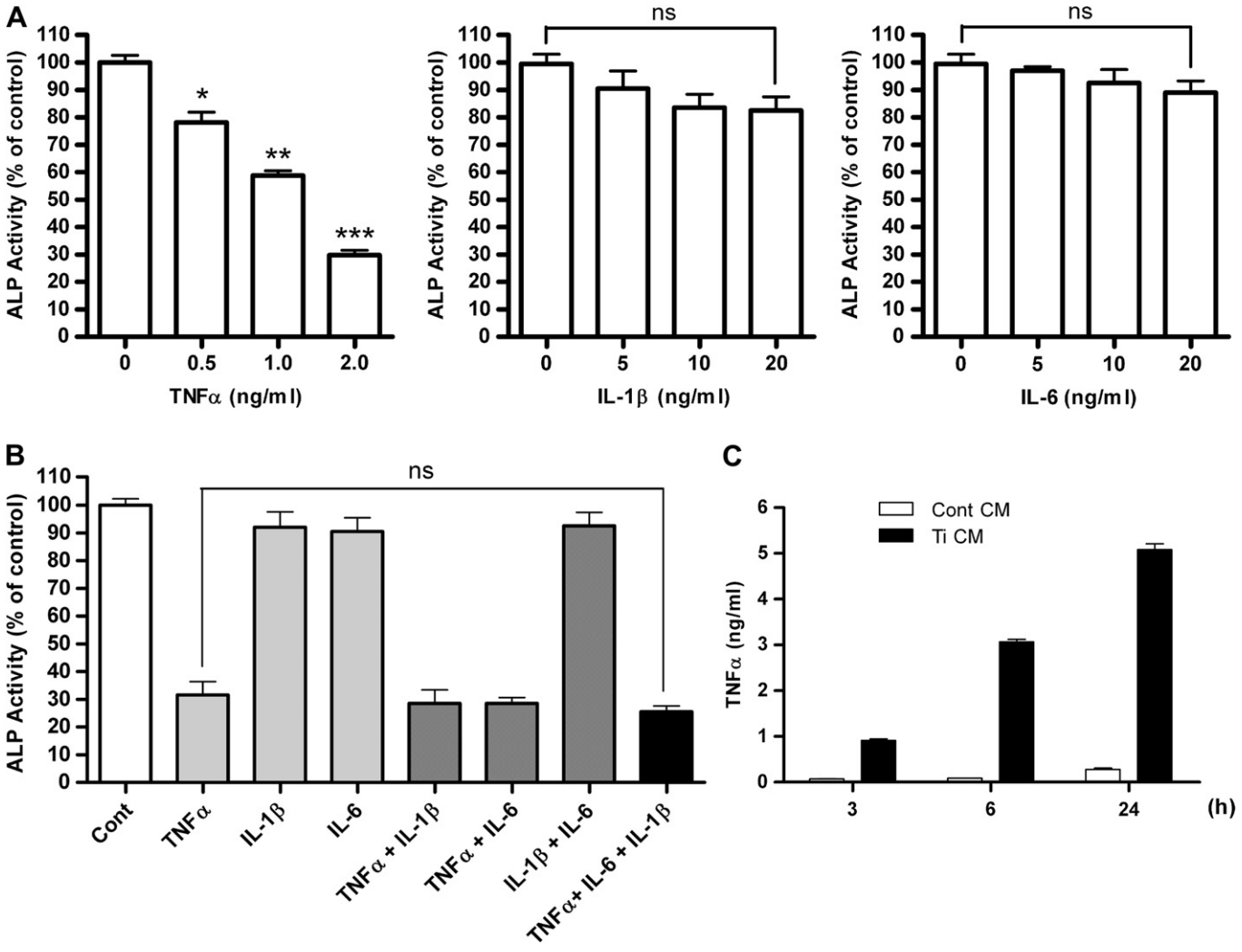

Fig. 3. Effects of secreted cytokines in Ti CM on ALP activity. (A) Recombinant proteins of TNFα, IL-1β, IL-6 were treated to MC3T3 E-1 cells at various doses as indicated. After 48 h, ALP activity was measured. (B) TNFα (2 ng/ml), IL-1β (10 ng/ml) and IL-6 (10 ng/ml) were subjected to MC3T3-E1 cells as combinatory treatment and ALP activity was analyzed after 48 h. (C) Ti CM was collected from RAW 264.7 cells after 3, 6, and 12 h of incubation with Ti particles (1:120). The concentrations of TNFα were measured by ELISA. Data are shown as the mean ± SD. Similar results were obtained in three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and ns (no significance).

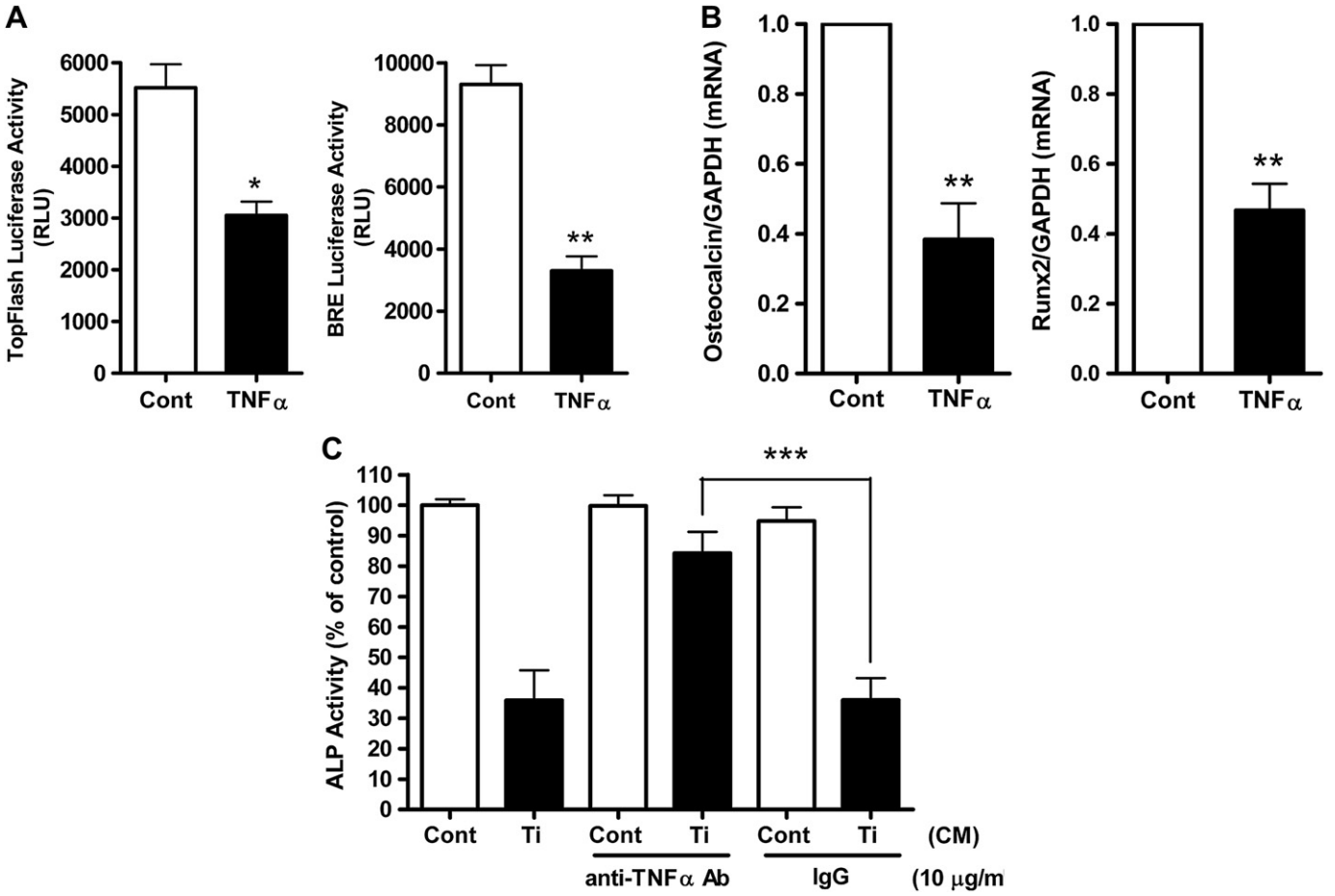

Fig. 4. TNFα mimics all the effects of Ti CM on osteogenic activity. (A) MC3T3 E-1 cells were transiently transfected with TopFlash-luc or BRE-luc reporter plasmids and treated with TNFα (2 ng/ml). After 48 h, luciferase activities were measured in cell lysates. (B) Following stimulation with TNFα (2 ng/ml) for 48 h, total cellular RNA was extracted. Expression of osteocalcin and Runx2 were analyzed by real-time RT-PCR. (C) Cont and Ti CM, pre-incubated with neutralizing antibody (10 μg/ml) against TNFα for 1 h, were treated to MC3T3 E-1 cells. As a control, isotype matched IgG was used. After 48 h, ALP activity was analyzed. Data are shown as the mean ± SD. Similar results were obtained in three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

the activation of NFκB signaling. To clarify the relationship of NFκB activation with suppressed osteogenic activity, we inhibited NFκB activity by using BAY11-7082, a pharmacological specific inhibitor for phosphorylation of IκBα (Fig. 5C). In the presence of BAY11-7082, Ti CM failed to suppress ALP activity to the extent seen in the absence of inhibitor. Pretreatment of BAY11-7082 for 1 h was sufficient to inhibit induction of NFκB activity (Fig. 5C).

3.6. Inhibition of exogenous stimulation with Wnt3a and Bmp2 in Ti CM treated osteprogenitors

WNT and BMP pathways are regulated by several processes. One of the known mechanisms is by secreted proteins, which act as extracellular antagonists. Noggin, chordin, sclerostin (SOST), and follistatin are BMP antagonists, while SOST, secreted frizzled-related protein (sFRP), Dickkopf (DKK), Wise and Wnt inhibitory factor (WIF) are Wnt antagonists [35–38]. Recently, TNFα has been shown to induce the expression of molecules which can suppress osteoblast differentiation such as the Wnt antagonists, DKK1 and SOST [16,39]. Therefore, we expected that osteoprogenitor cells in response to Ti CM or TNFα might induce the secretion of antagonists to regulate WNT and BMP pathways. To investigate this possibility, we tested whether exogenous stimulation can activate these pathways effectively in osteoprogenitor cells pre-incubated with Ti CM. For this, MC3T3 E-1 cells were incubated with Ti CM for 48 h and then stimulated with recombinant Wnt3a and Bmp2 proteins for additional 24 h before analyzing ALP activity. Ti CM abrogated inductive effect of Wnt3a or Bmp2 on ALP activity, whereas inducible activity was observed in Cont CM treated cells (Fig. 6A). When MC3T3 E-1 cells were incubated with TNFα instead of Ti CM, Wnt3a or Bmp2 again failed to induce ALP activity (Fig. 6B). Canonical WNT signaling acts through stabilizing of β-catenin whereas phosphorylation of Smad 1/5/8 is responsible for BMP signaling activity for osteoblast differentiation. These signaling molecules then translocate into the nucleus to regulate the expression of genes related to osteogenic activity, such as alkaline phosphatase and osteocalcin [36,40]. So, to precisely determine the inhibitory role on WNT and BMP signaling pathways in cells treated with Ti CM, the stability of β-catenin and phosphorylation of Smad 1/5/8 were detected by western blotting. We observed no induction of β-catenin stabilization in Ti CM treated cells even after stimulation of Wnt3a, whereas increased β-catenin stabilization was detected in Cont CM samples within 1 h of stimulation and thereafter (Fig. 6C). Similarly, induced phosphorylation of Smad 1/5/8 was detected within 15 min after stimulation of Bmp2 in cells treated with Cont CM, but no induction was observed in case of Ti CM (Fig. 6D).

3.7. Involvement of SOST for impeded osteogenic activity

To further investigate whether various negative regulators of WNT and BMP pathways are being up-regulated in MC3T3 E-1 cells

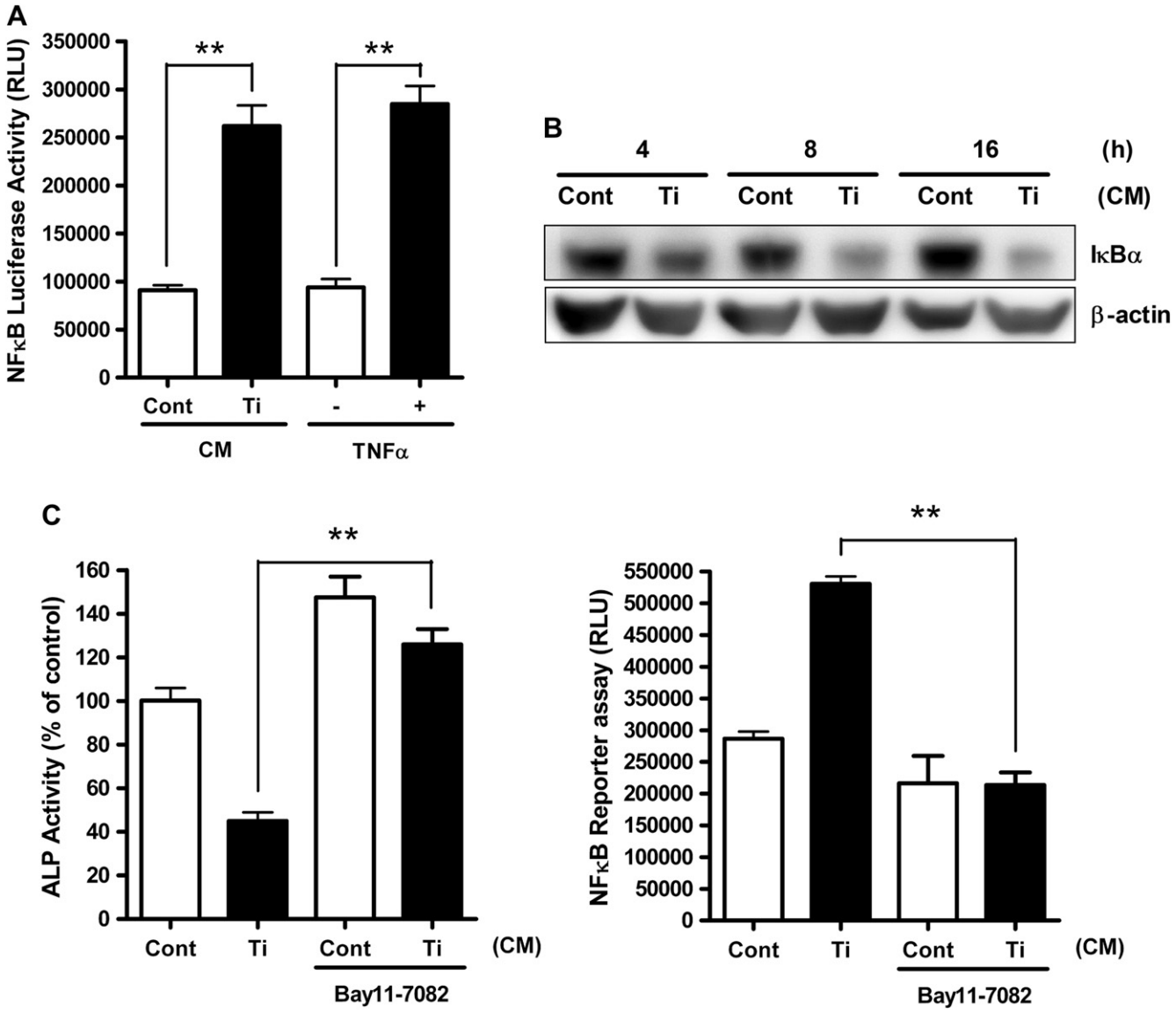

Fig. 5. Ti CM activates NFκB signaling to suppress ALP activity. (A) MC3T3 E-1 cells were transiently transfected with the NFκB-luc reporter plasmid and treated with Ti CM or TNFα (2 ng/ml). After 24 h, luciferase activity was measured in cell lysates. (B) After treatment of Ti CM for 4, 8 and 16 h, total cell lysates were collected and subjected to detect protein stability of IκBα by western blotting. β-Actin was measured as loading control. (C) MC3T3 E-1 cells were transiently transfected with the NFκB-luc reporter plasmid. After pretreatment of BAY11-7082 (2 μM) for 1 h, cells were subsequently treated with Ti CM for 48 h. The cells lysates were collected and were assayed for ALP and luciferase activity. Data are shown as the mean ± SD. Similar results were obtained in three independent experiments. $^{**}P < 0.01$.

by Ti CM or TNFα, we next examined the expression of known negative regulators by real-time RT-PCR. Among various antagonists screened for both WNT and BMP signaling pathways, mRNA expression level of DKK1, sFRP1 and sclerostin (SOST) was found to be elevated (Table 2 & Fig. 7A). Expression of DKK1 and sFRP1, inhibitors of WNT pathway, was found to be increased ~1.5 fold compared to SOST that showed ~2.5–3 fold increment, which has a well-known function in inhibiting both WNT [41] and BMP [42] signaling pathways, thus increasing the possibility of its involvement in our system as expected. However, none of these antagonists were over-expressed in RAW cells in response to Ti particles (data not shown). To examine the role of SOST instead of Ti CM on ALP activity, MC3T3 E-1 cells were transfected with SOST/pcDNA3.1 + plasmid encoding full length SOST. Over-expression of SOST showed suppression of ALP activity compared to transfection with GFP/pcDNA3.1 + control plasmid (Fig. 7B). Furthermore, to prove the role of SOST as a mediator of suppressed effect shown by Ti CM on ALP activity, MC3T3 E-1 cells were transfected with siRNA against SOST prior to treatment of Ti CM. The inhibition of SOST by siRNA partially restored the effect of Ti CM on ALP activity. Noticeably, the induced level of SOST by Ti CM was inhibited with siRNA transfection (Fig. 7C).

3.8. Suppressed osteogenic activity by Ti CM in human primary osteoblasts

To validate whether the above mentioned effects of Ti CM were also reproducible in another *in vitro* model of osteoblasts, cultures of primary human osteoblasts were established. Similar to MC3T3 E-1 cells, Ti CM or TNFα suppressed ALP activity as well as mRNA expression levels of osteocalcin and Runx2 in primary human osteoblasts. Moreover, Ti CM or TNFα induced the expression of SOST by ~2 fold (Fig. 8). Therefore, these results authenticate our previous results in MC3T3 E-1 cells treated with Ti CM or TNFα.

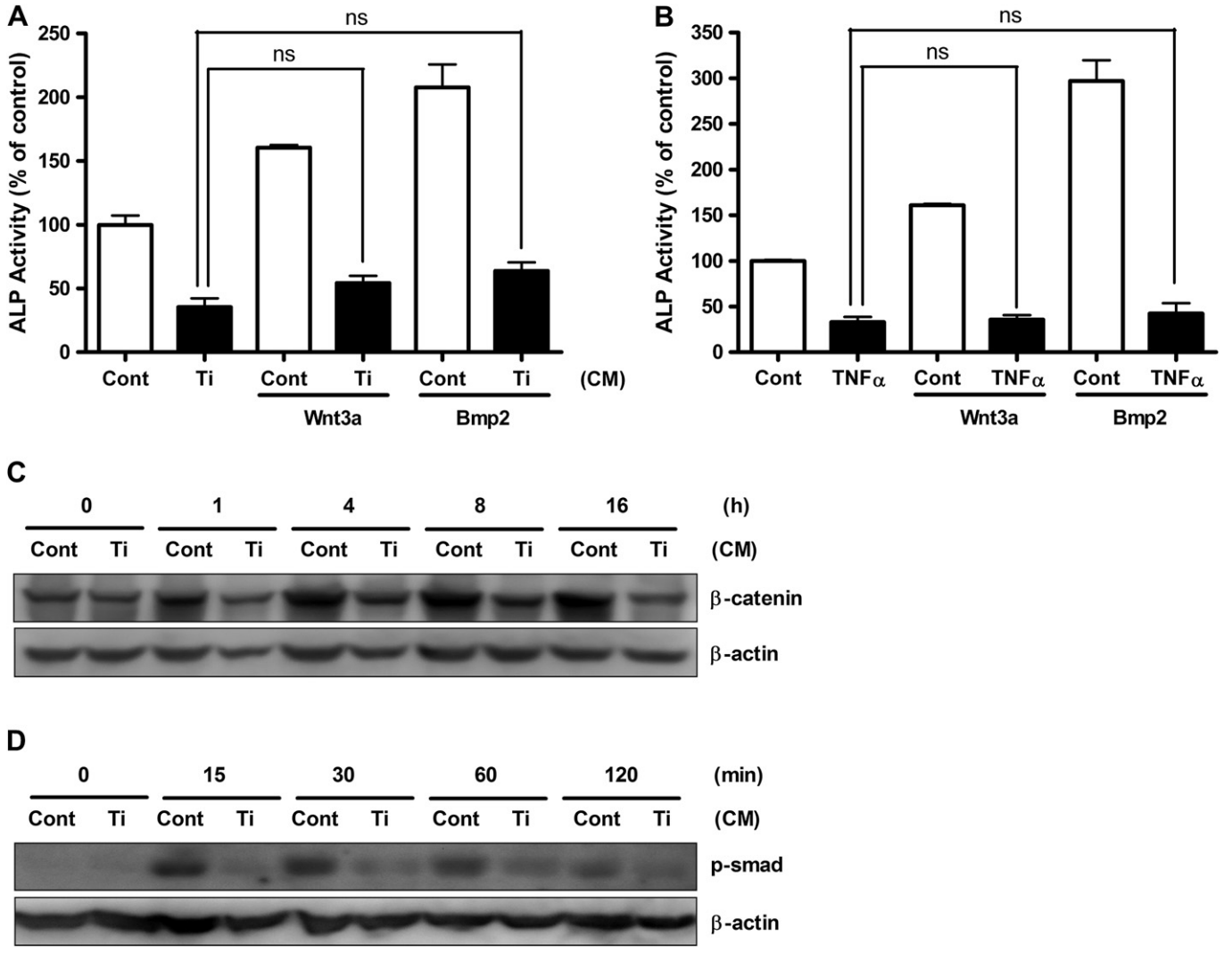

Fig. 6. Pre-incubation with Ti CM inhibits stimulation of exogenous Wnt3a or Bmp2. (A & B) After 48 h of treatment with Ti CM or TNFα (2 ng/ml), MC3T3 E-1 cells were stimulated by recombinant Wnt3a (50 ng/ml) or Bmp2 (100 ng/ml) for additional 24 h and assayed for ALP activity. (C & D) Similarly, after 48 h of treatment with Ti CM or TNFα (2 ng/ml), MC3T3 E-1 cells were stimulated by recombinant Wnt3a (50 ng/ml) or Bmp2 (100 ng/ml) for indicated time points and protein lysates were collected. The stabilization of β-catenin and phosphorylation of Smad1/5/8 were detected by western blotting. β-Actin was used as a loading control. Data are shown as the mean ± SD. Similar results were obtained in three independent experiments. ns (no significance).

4. Discussion

In the present study, we investigated the role of macrophages activated by Ti particles in modulating the osteogenic potential of osteprogenitor cells. Numerous macrophages exist at interfacial membrane of patients and become activated by interaction with wear debris with or without phagocytosis, releasing inflammatory mediators in microenvironment of the bone implants [43]. As activation of macrophages by wear particles can be reproduced with cultured macrophage lineage cells [44,45], RAW 264.7 cell line was used as a macrophage cell model and treated with Ti particles to collect conditioned medium. Ti particles were selected as wear debris in this study, since they are commonly generated from numerous orthopaedic prostheses, such as dental implants and spine pedicular screws, and also widely used for studies of inflammatory osteolysis. In accordance with above studies, treatment to RAW 264.7 cell with Ti particles also showed significant inflammatory response indicated by several fold increase in COX2 (Supplement 2B). Furthermore, conditioned medium collected from activated macrophages reduced various osteogenic

Table 2
Fold change of mRNA expression (normalized to GAPDH) for various antagonists of WNT and BMP signaling pathway in MC3T3 E-1 cells in response to Ti CM for 48 h.

Gene name	Average fold change
DKK1	1.47 ± 0.25*
DKK2	0.98 ± 0.30
DKK3	0.79 ± 0.28*
DKK4	0.88 ± 0.31
sFRP1	1.39 ± 0.29*
sFRP2	0.73 ± 0.31*
sFRP3	0.83 ± 0.36
sFRP4	0.72 ± 0.33*
sFRP5	0.80 ± 0.23*
Wise	0.69 ± 0.45*
WIF	0.67 ± 0.46*
SOST	2.43 ± 0.51*
Noggin	0.82 ± 0.33
Chordin	0.99 ± 0.28
Follistatin	0.87 ± 0.30

Data were generated from means of triplicate real-time RT-PCR reactions ± SD. *$p < 0.05$.

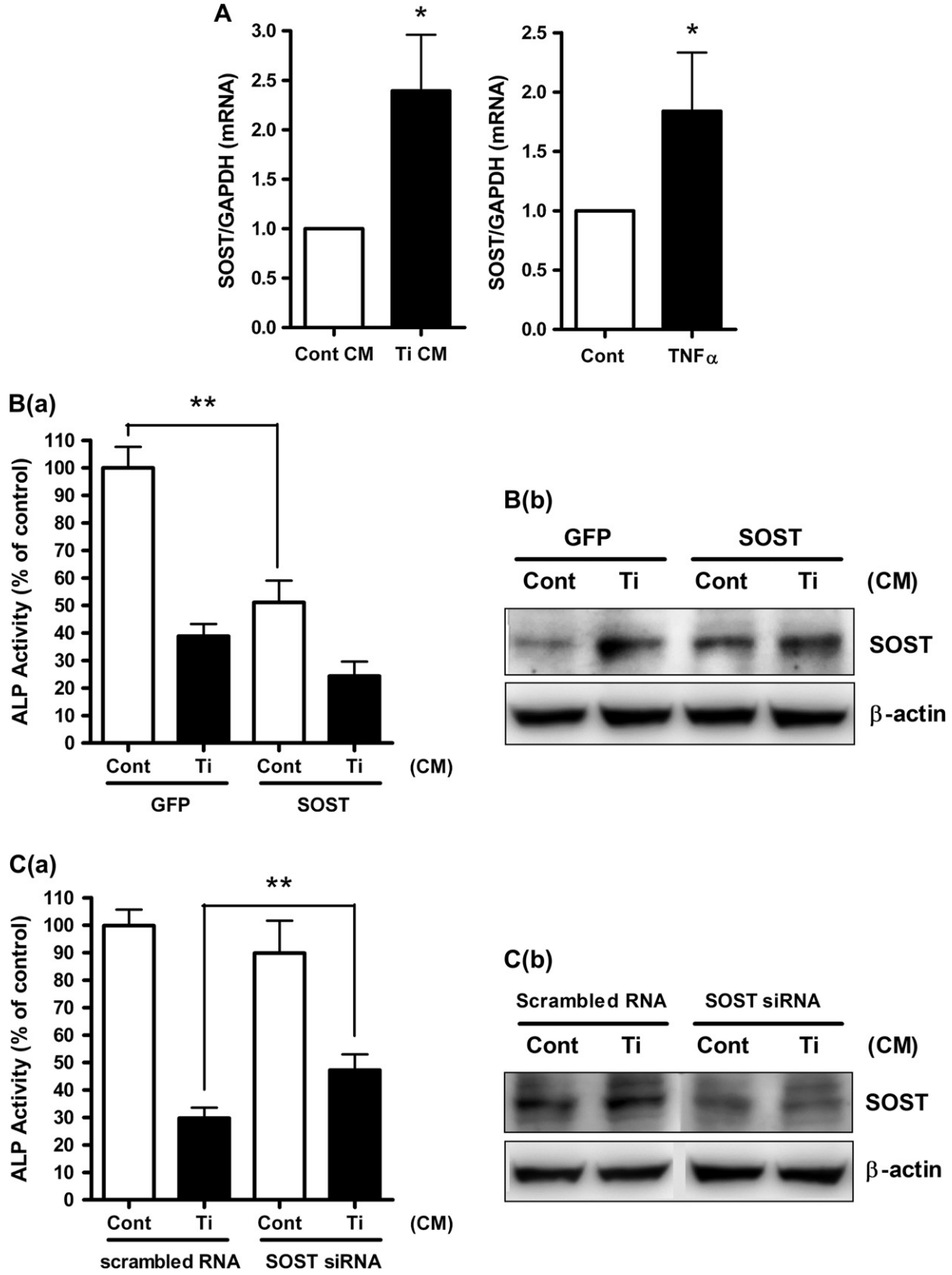

Fig. 7. Induction of SOST participates in suppressed ALP activity by Ti CM. (A) Expression of SOST was analyzed by real-time RT-PCR in response to Ti CM or TNFα (2 ng/ml) in MC3T3 E-1 cells. (B) For over-expression of SOST, MC3T3 E-1 cells were transiently transfected with SOST/pcDNA3.1 + plasmid, and after 48 h of Ti CM treatment, ALP activity was analyzed. GFP/pcDNA3.1 + plasmid were used as a control for transfection. With above samples, induced protein levels of SOST were detected by western blotting. (C) For knockdown of SOST, MC3T3 E-1 cells were transiently transfected with SOST siRNA. ALP activity was analyzed after 48 h of Ti CM treatment. Scrambled RNA was used as a control. With above samples, reduced protein levels of SOST were detected by western blotting. Data are shown as the mean ± SD. Similar results were obtained in three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$.

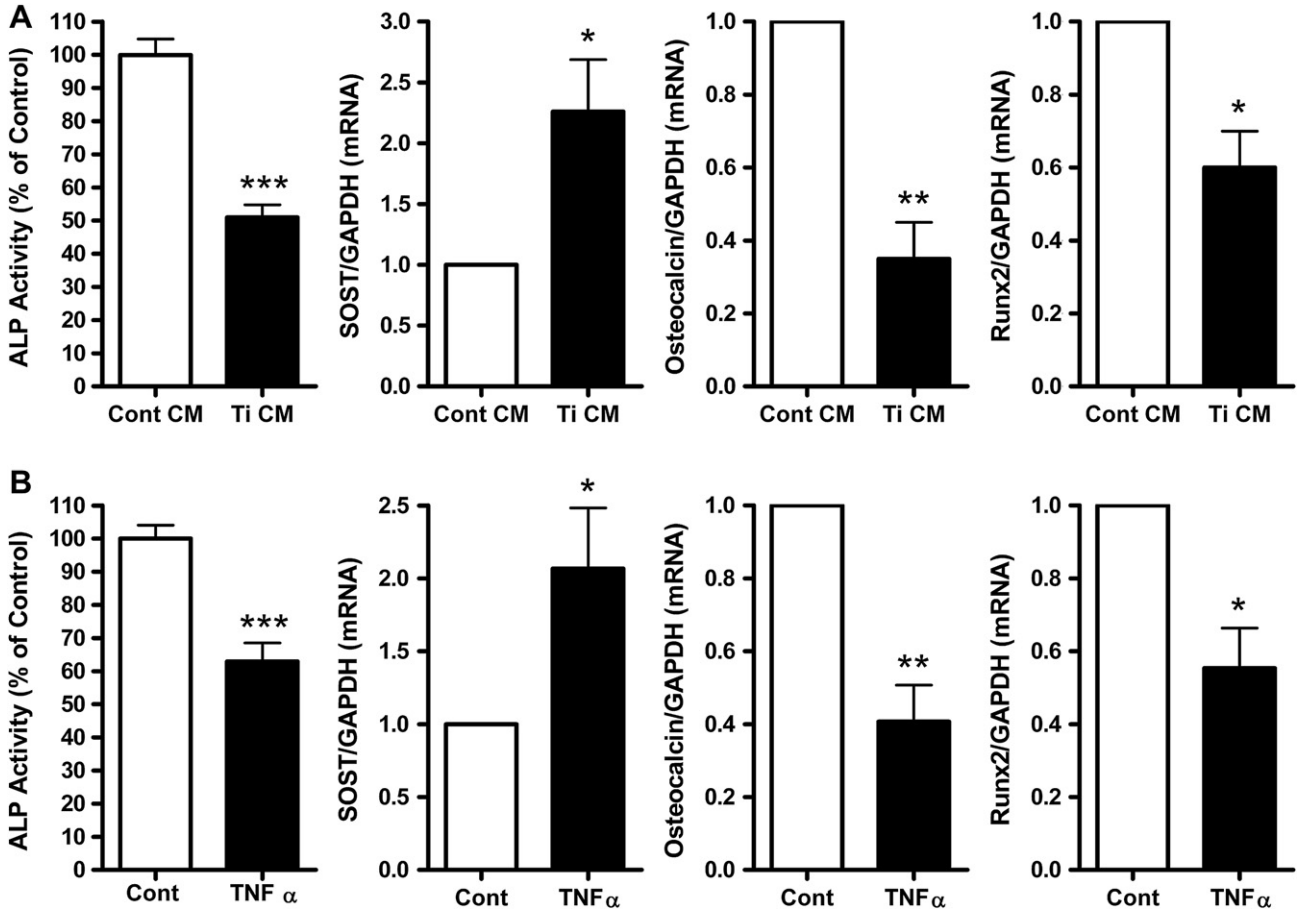

Fig. 8. Ti CM and TNFα impede differentiation potential of human primary osteoblasts. Human primary osteoblasts were treated with Ti CM (A) or TNFα (2 ng/ml) (B). After 48 h, cell lysates were collected and assayed for ALP activity. To determine the expression level of SOST, osteocalcin and Runx2, total cellular RNA was extracted from treated cells and subjected to analyze transcripts by real-time RT-PCR. The expression level of each gene is normalized to GAPDH. Data are shown as the mean ± SD. Similar results were obtained in three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

parameters such as ALP activity, collagen synthesis, matrix mineralization and expression of osteocalcin and Runx2 (Fig. 1). These results strongly suggest that secreted molecules from macrophages in response to Ti particle suppress both early and late stages of osteoblast differentiation. As the markers for differentiation of osteoblast are controlled by WNT and BMP signaling pathways [27], it is necessary to examine the effect of Ti CM on these two pathways. Our results showed impeded basal and induced WNT and BMP signaling activity of osteoprogenitor cells in response to Ti CM (Fig. 2), clarifying the inhibitory role of secretory molecules present in Ti CM on these signaling pathways.

Previous studies on the aspects of inflammatory response to implant debris have shown that a spectrum of cytokines are released by macrophages which leads to increased resorptive activity [22] but only few studies elucidated the role of released cytokines in inflammatory responses to decreased bone formation at mechanistic level. In view of this concept, we inspected the expression level of reported cytokines from activated macrophages and analyzed their effect on ALP activity. Consistent with previous reports [4,20], TNFα, IL-1β and IL-6 were over expressed in macrophages stimulated by Ti particles, but only TNFα mimicked the effects of Ti CM on ALP activity of osteoprogenitor cells as compared to IL-1β and IL-6 (Figs. 3and 4). Though IL-1β and IL-6 have been shown to affect osteoblast differentiation variably [29,31,46,47], no significant effect on ALP activity was found in our experimental system (Fig. 3). On the basis of above studies, it can be explained that IL-1β or IL-6 acts differently on cells at different stages of maturation. The analysis of TNFα effects showed reduction in expression of osteocalcin and Runx2 and process of matrix mineralization and collagen synthesis, similar to Ti CM, confirming its predominant role in impeded osteogenic activity induced by Ti CM. TNFα is a potent, multifunctional inflammatory cytokine that contributes to local and systemic bone loss in inflammatory bone diseases as well as estrogen deficiency [48,49]. In osteoblasts, a TNFα trimer binds to two receptor forms, type I and II, of which only type I eventually activates NFκB signaling along with other signaling pathways [50,51] contributing to the complexity of TNFα-induced effect on osteoblast differentiation, proliferation and apoptosis. Consistent with these studies, NFκB signaling was activated in osteoprogenitors treated with Ti CM (Fig. 5). Moreover, inhibition of NFκB signaling failed to suppress ALP activity, establishing critical role of NFκB signaling stimulated by Ti CM in impaired osteogenic activity. Recently, few reports have shown cross-talking between TNFα and WNT or BMP signaling pathways [52,53]. According to reporter assays for WNT and BMP signaling pathways, activities of these pathways were suppressed in osteoprogenitors treated with TNFα similar to Ti CM. These finding implicates that TNFα can modulate both of these signaling pathways during the process of inflammatory bone diseases, resulting impaired bone formation.

During differentiation process of osteoblast, WNT and BMP pathways are in tight regulation with secreted antagonists [54].

After 48 h of incubation with Ti CM, exogenous treatment with Wnt3a or Bmp2 could not show stimulatory effect on ALP activity as well as reporter activity. And also, β-catenin stabilization or phosphorylation of Smad 1/5/8 was not induced in osteoprogenitors incubated with Ti CM after stimulation of Wnt3a or Bmp2, respectively. Therefore, we hypothesized the possible involvement of secreted antagonist in regulatory mechanism of these pathways in osteoprogenitors after challenging with Ti CM (Fig. 6). To elucidate these results, expression of various antagonists for WNT and BMP signaling in response to Ti CM and TNFα were analyzed (Table 2). Among these, induction of SOST gene at mRNA and protein level was found to be significant (Fig. 7). A recent study emphasized that SOST expression is restricted in normal bone to mature osteocytes [55], however few studies has stated SOST induction in osteoblasts. SOST expression in mRNA level has been reported to be inducible by TNFα in human and mouse cell line models which includes MG-63 human osteosarcoma cells, an immature human osteoblast model [56], and undifferentiated mouse MC3T3-E1 cells, an immature osteoblast cell line model [57]. Taken together, these data signify that immature osteoblasts may be stimulated to express SOST under inflammatory conditions, probably contributing to the bone forming defects. In addition to suppression of bone formation [58], SOST has been shown to inhibit both WNT and BMP signaling pathways [59]. For addressing the accountability of SOST in the current study, over-expression of SOST led to decreased ALP activity (Fig. 7B), showing its suppressive effect similar to Ti CM. Whereas, inhibition of SOST expression by siRNA approach resulted in partial recovery of suppressed ALP activity (Fig. 7C). These results strongly imply that the induced expression of SOST contributes to impeded osteoblast differentiation process. Therefore, we suggest that TNFα may accomplishes impeded osteogenic activity by inducing antagonists such as SOST, abrogating WNT and BMP signaling in inflammatory osteolysis. Ti CM showed similar effects in human primary osteoblasts substantiating our finding in MC3T3 E-1 preosteoblast model (Fig. 8).

However, as complete recovery of ALP activity was not restored after inhibition of SOST, possibility of other mechanism remains to be studied. Recent reports have identified TNFα-mediated induction of DKK1 in a mouse model of inflammatory arthritis and TNFα transgenic mice [16]. Although, we did not observe significant effect of Ti CM or TNFα on upregulation of DKK1 in MC3T3 E-1 cells, we cannot rule out the possible participation of other antagonists such as DKK1 and sFRP1 in the bone forming activity. Other possibility that remains to be elucidated is the change of susceptibility for the stimulation of WNT and BMP signaling pathways in osteoprogenitors treated with Ti CM. TNFα has been reported to differentially modulate BMP receptors both at post-transcriptional and post-translational levels, diminishing the response to Bmp2 *in vitro* [60]. Thus, the possible role of TNFα on modulation of receptor level for WNT and BMP must be studied in details to further explain the regulatory mechanism of impaired osteogenic activity. Taken together, the data presented here clearly demonstrates that the macrophages on direct interaction with Ti particles are able to modulate osteogenic activity of osteprogenitor cells. This is achieved by releasing proinflammatory cytokines such as TNFα which predominantly impedes bone forming potential of osteoprogenitors by regulating WNT and BMP signaling activity. TNFα plays a role in inducing the expression of SOST, which is able to regulate WNT and BMP signaling pathways, impairing osteoblast differentiation process. Our findings extend the repertoire of TNFα secreted from macrophages to impaired bone forming activity other than induced bone resorption by stimulating osteoclastogenesis in inflammatory bone diseases including osteolysis mediated by wear particles.

5. Conclusion

In summary, this study clearly shows that (1) macrophages in response to Ti particles contribute to suppressed bone forming activity of osteoprogenitors, seen in inflammatory osteolysis, (2) TNFα play a main role in impaired osteogenic activity of osteoprogenitors via regulating WNT and BMP signaling pathways, and (3) autocrine induction of SOST by TNFα is able to hamper osteoblast differentiation process by annulling WNT and BMP signaling.

These observations propose a regulatory mechanism of bone defects that are identified in inflammatory osteolysis such as periprosthetic osteolysis and rheumatoid arthritis, and provide further evidence that SOST regulated by TNFα may be a suitable therapeutic target for treatment of impaired bone forming activity in inflammatory particle diseases.

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Appendix A. Supplementary data

Supplementary data related to this article can be found online at doi:10.1016/j.biomaterials.2012.03.005.

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