Biochemical and Biophysical Research Communications 389 (2009) 28–33

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

# Enhanced inhibitory effects of a novel CpG motif on osteoclast differentiation via TREM-2 down-regulation

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ARTICLE INFO

*Article history:*
Received 6 August 2009
Available online 19 August 2009

*Keywords:*
CpG-ODN
Osteoclastogenesis
TLR9
TREM-2
NFATc1

ABSTRACT

Recognition of oligodeoxynucleotides containing CpG motifs (CpG-ODNs) by toll-like receptor 9 (TLR9) inhibits RANKL-induced osteoclastogenesis from precursors. This inhibitory effect suggests the possibility of using this strategy to block pathological bone loss. However, the enhancing effect of CpG-ODNs on OC formation from RANKL-primed pre-osteoclasts (pOCs) has hampered their clinical use. In this report, we developed a CpG-KSK13 oligonucleotide with an alternative CpG motif, and tested its effect on osteoclastogenesis in comparison with previously used murine CpG motif (CpG-1826) or human CpG motif (CpG-2006) oligonucleotides. Murine CpG-1826 inhibited RANKL-induced OC formation from BMMs but not from RANKL-primed pOCs, while CpG-KSK13 treatment strongly inhibited OC formation from both BMM and primed pOC cells. CpG-KSK13 also showed a potent inhibitory effect on human OC differentiation using peripheral blood mononuclear cells (PBMCs), which was in contrast to the species-specific response of murine CpG-1826 or human CpG-2006. Moreover, CpG-KSK13 effectively inhibited NFATc1 activity, but not NF-κB or AP-1 activity, and decreased TREM-2 promoter activity and subsequent surface expression of the TREM-2 protein induced by M-CSF and RANKL. These results demonstrate that the recognition of CpG-KSK13 via TLR9 inhibits osteoclastogenesis by down-regulating TREM-2 expression. Thus, our findings provide evidence for the potential use of CpG-KSK13 as an anti-osteoclastogenic agent for human and for pre-clinical animals.



## Introduction

Bone resorbing osteoclasts (OCs) are highly specialized multinucleated giant cells that are differentiated from the monocyte/macrophage lineage of hematopoietic cells [1,2]. Two factors, macrophage colony-stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANKL), are required for osteoclast (OC) formation, survival, and function [3]. In addition, recent evidence has suggested that immunoreceptors, such as osteoclast-associated receptor (OSCAR) and triggering receptor expressed by myeloid cells (TREM)-2, which are associated with the immunoreceptor tyrosine-based activation motif (ITAM) containing adaptor proteins, FcE receptor I γ chain (FcRγ) or DAP12, are important for osteoclastogenesis, as well as OC maturation and function *in vitro* and *in vivo* [4–7]. These OSCAR-FcRγ and TREM-2-DAP12 signaling complexes act as the co-stimulatory signals on OC precursors and are required for RANKL-mediated activation of calcium signaling, which leads to NFATc1 activation during osteoclastogenesis [7].

Toll-like receptors (TLRs) on host cells are responsible for the innate immune response by recognizing pathogen-associated microbial molecules [8]. This recognition occurs in TLR-expressing cells of hematopoietic origin as well as immune cells, which provides a mechanism by which these factors can modulate cellular response [9]. In line with this notion, bone resorbing OCs originating from hematopoietic cells are modulated by TLR signaling during microbial infection [10]. The pattern recognition in OC precursors of various ligands including peptidoglycan, poly (I:C) dsRNA, bacterial lipopolysaccharide (LPS), and unmethylated CpG motifs on bacterial DNA by TLR2, 3, 4, and 9, respectively, inhibit OC formation

*Abbreviations:* BMM, bone marrow macrophages; CpG-ODN, CpG oligodeoxynucleotides; OCs, osteoclasts; PBMC, peripheral blood monocytic cells; RANKL, receptor activator of NF-κB ligand; TLR9, toll-like receptor 9; TREM-2, triggering receptor expressed on myeloid cells

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doi:10.1016/j.bbrc.2009.08.080

[10]. These modulations via TLR interactions can be expected to be regulatory factors controlling pathological bone loss.

Particular attention has focused on TLR9 as a clinical target for modulating the immunostimulatory effect mediated by synthetic oligodeoxynucleotides containing CpG motifs (CpG-ODNs) against cancer, allergy, and autoimmune diseases [11,12]. Because of its TLR9 dependent modulatory effect on osteoclastogenesis [13], CpG immunotherapy has been suggested as a source of therapeutics for bone-degenerating diseases. However, the clinical application of CpG-ODNs has been deterred by a limitation; the dual opposing effects of CpG-ODNs on osteoclastogenesis, primarily an inhibitory effect on OC precursors and a stimulatory effect on pre-osteoclasts (pOCs) primed by RANKL [10,13–15]. The dual effects are due to the decrease in M-CSF receptors on OC precursors and the increase in TNF-α and RANKL expression in RANKL-primed cells [16,17]. An *in vivo* study showed that CpG-ODNs induce OC-like cell formation through a direct effect on RANKL-primed cells and thereby increase bone loss in diseases such as periodontitis and rheumatoid arthritis [16,18]. More importantly, since increased RANKL secretion by cancer cells is highly associated with osteolysis in bone tumor [16], the undesirable bone loss can be occurred in the immunotherapy using CpG-ODNs in the treatment of cancer. In this regard, it is important to predict *in vivo* outcomes of CpG immunotherapy affecting human bones using the data from pre-clinical animals. However, the species restriction of CpG-ODNs has posed difficulty in extrapolating animal data to human [19]. Therefore, it is desirable to develop an immunotherapeutic approach using new CpG-ODNs that are more effective in OC suppression irrespective of species.

In this study, we have developed a new CpG-ODN, CpG-KSK13, which showed an enhanced inhibitory effect on murine bone marrow macrophages (BMMs)-derived and human peripheral blood monocyte (PBMC)-derived OC formation. This inhibitory effect was due to the down-regulation of TREM-2 expression in osteoclast precursors, which regulates NFATc1 activity. These results provide the possibility of utilizing this new CpG-ODN, CpG-KSK13, in the clinical setting as an anti-osteoclastogenic therapeutic agent.

## Materials and methods

*Reagents and antibodies.* RANKL and M-CSF were purchased from PeproTech (Rocky Hill, NJ). Lipofectamine 2000™ was from Invitrogen Life Technologies (Carlsbad, CA). The TLR9 antibody was from Cell Signaling (San Diego, USA). The TREM-1 and TREM-2 antibodies were from Abcam (Cambridge, UK). The TRAP staining kit (387A), actin antibody and all other reagents were from Sigma–Aldrich (St. Louis, MO). CpG-ODNs were completely phosphorothioate-modified as previously described [20,21] and were provided by MWG-Biotech AG (Ebersberg, Germany). The sequences used in this study are as follows: non-CpG, 5′-TCC AGG ACT TCT CTC AGG TT-3′; human CpG-2006, 5′-TCG TCG TTT TGT CGT TTT GTC GTT-3′; murine CpG-1826, 5′-TCC ATG ACG TTC CTG ACG GTT-3′; and new CpG-KSK13, 5′-TCG TCG TTT TCG TCG TCG TTTT-3′. All CpG-ODNs contained <0.1 EU/ml of endotoxin as determined by the Limulus assay (Bio-Whittaker, Walkersville, MD, USA).

*Osteoclast differentiation.* Mouse bone marrow (BM)- or human peripheral blood mononuclear cell (PMBC)-derived OCs were generated as previously described [22]. Non-adherent bone marrow cells were cultured with M-CSF (30 ng/ml) for 3 days; the resulting bone marrow macrophages (BMMs) were then used as OC precursors. Cells were cultured in α-MEM/10% FBS with M-CSF (30 ng/ml) plus RANKL (100 ng/ml) for 3 days to obtain pOCs. Generation of mature OCs was achieved by additional culturing for 2 days in the same conditions. PBMCs were obtained from peripheral blood of healthy donors and cultured with M-CSF (100 ng/ml) plus RANKL (100 ng/ml) for 7–9 days.

*Tartrate-resistant acid phosphatase (TRAP) staining.* OC formation was determined by staining for TRAP activity using the Leukocyte Acid Phosphatase Assay kit (Sigma). Cells were fixed with 3.7% formaldehyde for 5 min and stained according to the manufacturer's instruction.

*Luciferase reporter assay.* To examine the transcriptional activity of AP-1, NF-κB and NFATc1 or the OSCAR and TREM-2 promoter activity, murine BMMs were transiently transfected with the corresponding luciferase reporter constructs. Transfected cells were pretreated with or without a non-functional negative control CpG motif ODN (nCpG) or a murine-specific CpG-ODN (CpG-1826) for 24 h and subsequently incubated with M-CSF (30 ng/ml) plus RANKL (100 ng/ml) for 24 h. Cells were lysed in Glo Lysis Buffer (Promega) and the luciferase activity was measured using a luminescence counter.

*Flow cytometry analysis.* To determine the surface expression of TREM-1 and TREM-2, cells were incubated with the indicated Abs for 20 min on ice and washed 3 times with PBS. Flow cytometric analysis was performed using a FACSCalibur flow cytometer. CellQuest software (Becton Dickinson) was used for data acquisition and analysis.

*Statistical analysis.* Each experiment was performed 3–5 times. All quantitative experiments were performed in triplicate and the data are shown as means ± SD. Statistical significance was analyzed by unpaired Student *t* test.

## Results

### *Potent inhibition of CpG-KSK13 on osteoclast differentiation from mouse precursors*

Several studies have shown opposing effects of CpG-ODNs via TLR9 interaction on OC differentiation: an inhibitory effect on OC precursors and a stimulatory effect on RANKL-primed cells [13–15]. To explore the effect of a new CpG-ODN, CpG-KSK13, on OC differentiation, we first examined the effect of CpG-KSK13 on osteoglastogenesis using two stage cells of BMMs or RANKL-primed pOCs and compared it with the known effect of a previous murine-specific CpG-ODNs (CpG-1826) that showed a similar dual effect on osteoclastogenesis [14]. As shown in Fig. 1A, non-CpG-ODNs with a non-functional CpG motif, murine CpG-ODNs, CpG-1826, or our new CpG-KSK13 was added to BMMs at day 0 (Fig. 1A, I) or to pOCs at day 3 (Fig. 1A, II) along with M-CSF and RANKL. Consistent with other findings [13,14], we found that the treatment with CpG-1826 (1826) or CpG-KSK13 (KSK13) inhibited RANKL-induced OC formation in murine BMMs (Fig. 1B, upper (I), and C). In contrast, CpG-KSK13 exerted an inhibitory effect on OC differentiation in RANKL-primed pOCs, which was distinct from the stimulatory effect of murine CpG-1826 in those cells (Fig. 1C, lower (II), and D). These data indicate that the inhibitory effect of CpG-KSK13 on osteoclastogenesis is more potent than that of previous CpG-ODNs.

### *KSK-CpG13 inhibits both murine BMM- and human PBMC-derived osteoclastogenesis*

Since the effects of CpG-ODNs have been reported to be species-specific [19,23], it would be advantageous to develop alternative CpGs that show similar efficacy on both murine and human systems, in order to consider their use in the clinical setting. To determine whether the effect of CpG-KSK13 was species-restricted, we compared its potency with the effect of previous CpG-ODNs on murine BMM- or human PBMC-derived osteoclastogenesis using three different CpG-ODNs: human prototype CpG-2006 (2006), murine CpG-1826 (1826), and our new CpG-KSK13 (KSK13). Mur-

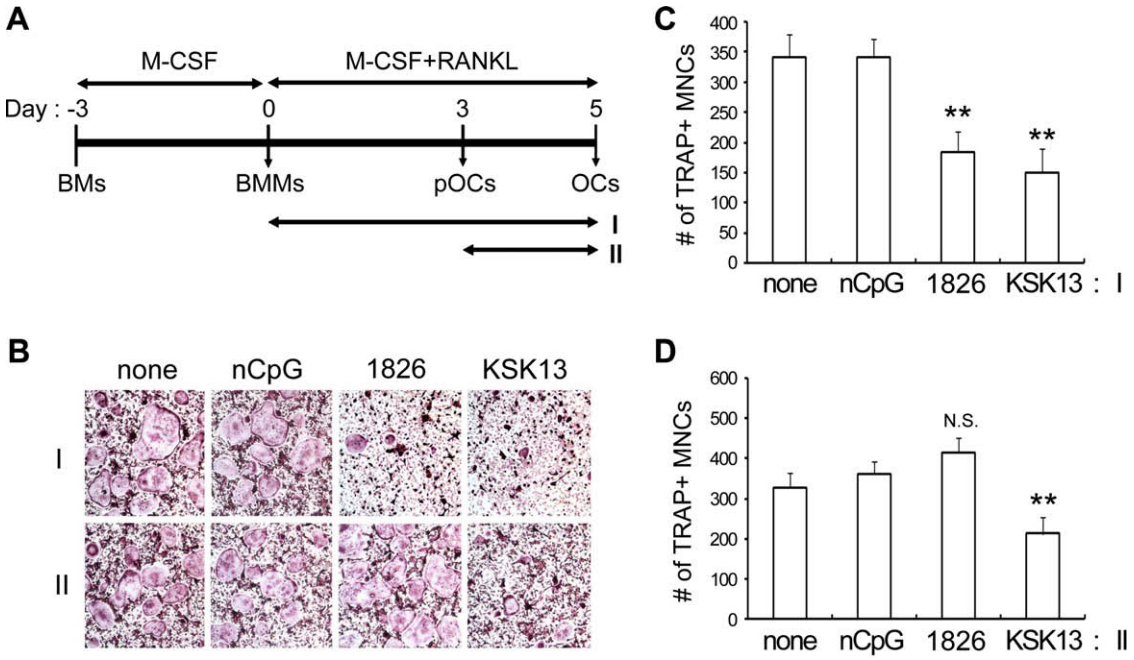


**Fig. 1.** The effects of a new synthetic CpG-ODN, CpG-KSK13, on RANKL-induced osteoclastogenesis. (A) Experimental scheme of osteoclastogenesis using mouse BMMs. Non-functional negative CpG motif (nCpG), murine-specific CpG-1826 (1826) or new synthetic CpG-ODNs, CpG-KSK13 (KSK13), was added at the stage of BMMs (I) or RANKL-primed pOCs (II) during osteoclastogenesis. (B–D) Effects of CpG-KSK13 on osteoclastic differentiation. BMMs (I) or pOCs (II) were treated with nCpG (10 μg/ml), CpG-1826 (1826) (10 μg/ml) or CpG-KSK13 (KSK13) (10 μg/ml) and cultured with M-CSF (30 ng/ml) plus RANKL (100 ng/ml) to generate OCs. The cells were fixed and stained for TRAP (B). $TRAP^+$ multinuclear cells with more than three nuclei were scored (C,D). N.S, not significant; $^{**}P < 0.01$ as compared with nCpG-treated group.

ine CpG-1826 or human CpG-2006 exerted only the inhibitory effect on osteoclastogenesis from the mouse BMMs (Fig. 2A) or human PBMCs (Fig. 2B), respectively. However, we found that CpG-KSK13 dramatically inhibited OC formation in both murine BMMs and human PBMCs (Fig. 2, A and B).

*TLR9 activation inhibits NFATc1 activity by RANKL in BMMs*

In this study, CpG-KSK13 showed an inhibitory effect on RANKL-induced osteoclastogenesis from BMMs and pOCs. To further define the inhibitory mechanism of CpG-KSK13 on osteoclastogenesis, we tested whether the activation of TLR9 recognized by CpG motifs was involved in RANKL-induced activation of transcription factors such as NF-κB, AP-1, and NFATc1. We found a ~9-, ~60-, or ~12-fold increase in AP-1- (Fig. 3A), NF-κB- (Fig. 3B), and NFATc1-mediated transcriptional activity (Fig. 3C), respectively, in transfected BMMs treated for 24 h with nCpG in the presence of M-CSF and RANKL compared to the untreated cells. Notably, CpG-1826 or CpG-KSK13 significantly inhibited M-CSF plus RANKL-induced transcriptional activity of NFATc1 (Fig. 3C), but did not inhibit the transcriptional activity of AP-1 (Fig. 3A) and NF-κB (Fig. 3B). These results demonstrate that CpG-KSK13 treatment inhibits NFATc1 activity, resulting in the failure of OC formation.

*TLR9 activation decrease surface expression of TREM-2*

The cooperative signaling from co-receptors such as OSCAR and TREM-2 is important for NFATc1 activation through cell-to-cell contact of OC precursors [4]. To define the possible mechanisms by which TLR9 activation by CpG-1826 or CpG-KSK13 controlled NFATc1 activity in BMMs, we examined the effect of those CpG-ODNs on OSCAR- and TREM-2 expression in our murine OC differentiation system. We found that M-CSF plus RANKL stimulation for 24 h in the presence of nCpG induced OSCAR promoter activity by

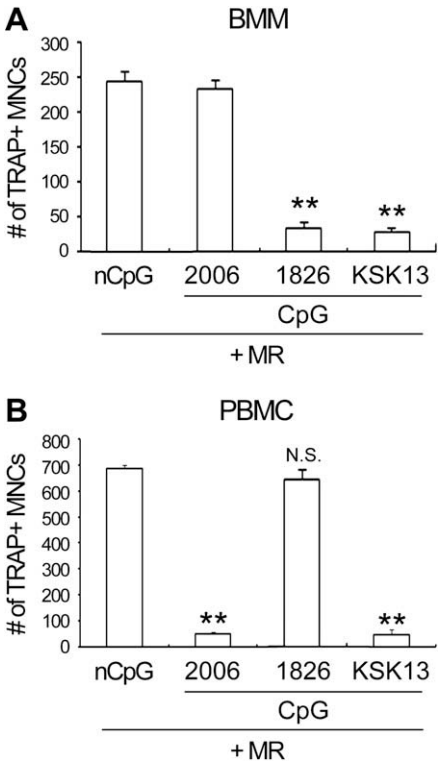


**Fig. 2.** Inhibitory effects of CpG-KSK13 on murine BMM- and human PBMC-derived osteoclastogenesis. (A,B) Inhibition of BMM- or PBMCs-derived osteoclast formation by CpG-KSK13. OCs were generated from BMMs (A) and human peripheral blood monocytes (PBMCs) (B) in the presence of nCpG or human specific CpG-2006 (2006), murine-specific CpG-1826 (1826), or the new synthetic CpG-KSK13 (KSK13). After TRAP staining, $TRAP^+$ multinuclear cells with more than three nuclei were scored. N.S, not significant; $^{**}P < 0.01$ as compared with nCpG-treated group. +MR, M-CSF plus RANKL.

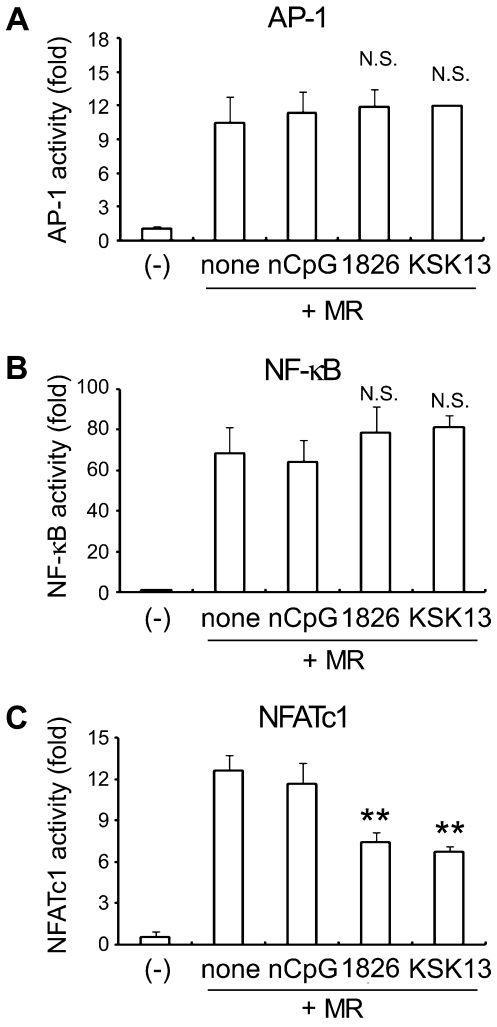


**Fig. 3.** Effects of CpG-KSK13 on transcription factor activation in osteoclast precursors. (A–C) BMMs were transfected with a luciferase plasmid carrying the binding site for the indicated transcription factor. After 24 h, cells were pre-treated with or without nCpG, CpG-1826 (1826) or CpG-KSK13 (KSK13) for 30 min and then additionally incubated with M-CSF (30 ng/ml) plus RANKL (100 ng/ml) for 24 h. Cells were lysed and then subjected to the luciferase assay. N.S, not significant; ** $P < 0.01$ as compared with nCpG-treated group. +MR, M-CSF plus RANKL.

~40-fold (Fig. 4A) and TREM-2 promoter activity by ~11-fold (Fig. 4B) in transfected BMMs compared to the untreated cells. Both CpG-1826 and CpG-KSK13 treatment of M-CSF plus RANKL-stimulated BMMs did not affect the OSCAR promoter activity (Fig. 4A). In contrast, CpG-1826 and CpG-KSK13 treatment significantly reduced TREM-2 promoter activity, as compared to the nCpG-treated cells (Fig. 4B). TREM-2 promoter activity increased by ~9-fold and by ~3-fold, respectively, after treatment with CpG-1826 and CpG-KSK13, which was less than that of the ~11-fold induced by nCpG treatment (Fig. 4A and B). Interestingly, we found that the inhibitory effect of CpG-KSK13 treatment on TREM-2 promoter activity was more potent than that of CpG-1826 (Fig. 4B).

In correlation with TREM-2 promoter activity, the surface expression of TREM-2 in BMMs was up-regulated by M-CSF plus RANKL treatment for 24 and 48 h in a time-dependent manner (Fig. 4C, right), which was similar to the upregulation of TREM-1 surface expression (Fig. 4C, left). CpG-1826 and CpG-KSK13 treatment did not have an effect on the M-CSF plus RANKL-stimulated TREM-1 surface expression (Fig. 4D, left). Strikingly, treatment with KSK-CpG13 caused a marked decrease in TREM-2 surface expression after 48 h stimulation with M-CSF and RANKL compared to the untreated or nCpG-treated cells; CpG-1826 treatment showed less reduction (Fig. 4D, right). These results suggest that CpG-KSK13 inhibits osteoclastogenesis through down-regulation of TREM-2 expression on OC precursors.

## Discussion

In this study, we found potent inhibitory effects of a new synthetic CpG-ODN, CpG-KSK13, on OC formation from both OC precursors and RANKL-primed pOCs (Fig. 1). The effect of CpG-KSK13 shows distinct characteristics from that of previous CpG-ODNs, suggesting an advantage of CpG-KSK13 to overcome the opposing effects of various CpG-ODNs on OC differentiation. Moreover, the similar efficacy profile of CpG-KSK13 on murine and human system (Fig. 2) enables one to predict the physiological response in human based on results from *in vivo* pre-clinical tests of murine bone loss models. It may be helpful to predict the efficacy of CpG-ODN as potential anti-osteoclastogenic therapeutics.

To define the underlying mechanism for the potent inhibitory effect of CpG-KSK13 during osteoclastogenesis, we focused on the activation status of transcription factors in OC precursors. It is well established that RANKL–RANK signaling is crucial for the activation of NF-κB, AP-1, and NFATc1 transcription factors in OC precursors [24]. As shown in Fig. 3, CpG-KSK13 treatment led to no difference in AP-1 activity (Fig. 3A), an increase in NF-κB activity (Fig. 3B), and a decrease in NFATc1 activity (Fig. 3C) in response to M-CSF plus RANKL. It is interesting that CpG-KSK13 can inhibit osteoclastogenesis, even while CpG-KSK13 activates NF-κB by RANKL treatment (Fig. 3B), which is consistent with the previous findings using CpG-1826 [14]. Based on our findings, CpG-KSK13 probably blocked osteoclastogenesis by suppressing NFATc1 activity known to be essential for the OC differentiation-related gene expression [24].

How does CpG-KSK13 decrease NFATc1 activity? A previous report supported a positive feedback loop in the immunoreceptor-NFATc1 pathway during osteoclastogenesis and proposed two means of NFATc1 activation [25]. One explanation of NFATc1 activation is that RANKL-induced NFATc1 activity induces OSCAR expression during osteoclastogenesis by binding to the OSCAR promoter. Another explanation is that the cooperative signaling by immunoreceptors with RANKL–RANK signaling on OC precursors that is required for selective NFATc1 activation. In this context, we hypothesized that CpG-KSK13 might influence the expression of OSCAR or TREM-2. As shown in Fig. 3A, CpG-KSK13 decreased TREM-2 promoter activity but not OSCAR promoter activity, allowing us to rule out the former possibility. One report showed that RANKL stimulation with M-CSF failed to induce TREM-2 mRNA expression [25]. In contrast, it was reported that the surface expression of TREM-2 was increased by M-CSF treatment in murine BMMs, an effect that was made more robust by co-treatment of M-CSF and RANKL [26]. In human PBMCs, M-CSF alone increased TREM-2 surface expression [27]. This knowledge has led to us to focus on whether CpG-KSK13 regulates the surface expression of TREM-2 in our culture system. As expected, M-CSF plus RANKL treatment increased TREM-2 surface expression to a greater degree (Fig. 4C) than M-CSF alone (data not shown). Furthermore, its expression was suppressed by CpG-KSK13 treatment (Fig. 4D). Therefore, it is conceivable that TLR9 activation by CpG-KSK13 treatment down-regulates TREM-2 expression leading to subsequent suppression of NFATc1 activity and inhibition of OC differentiation.

A critical role for TREM-2 in functional multinucleated OCs formation is suggested from the evidence showing the impaired bone resorptive activity in TREM-2-deficient patients [27]. Even while

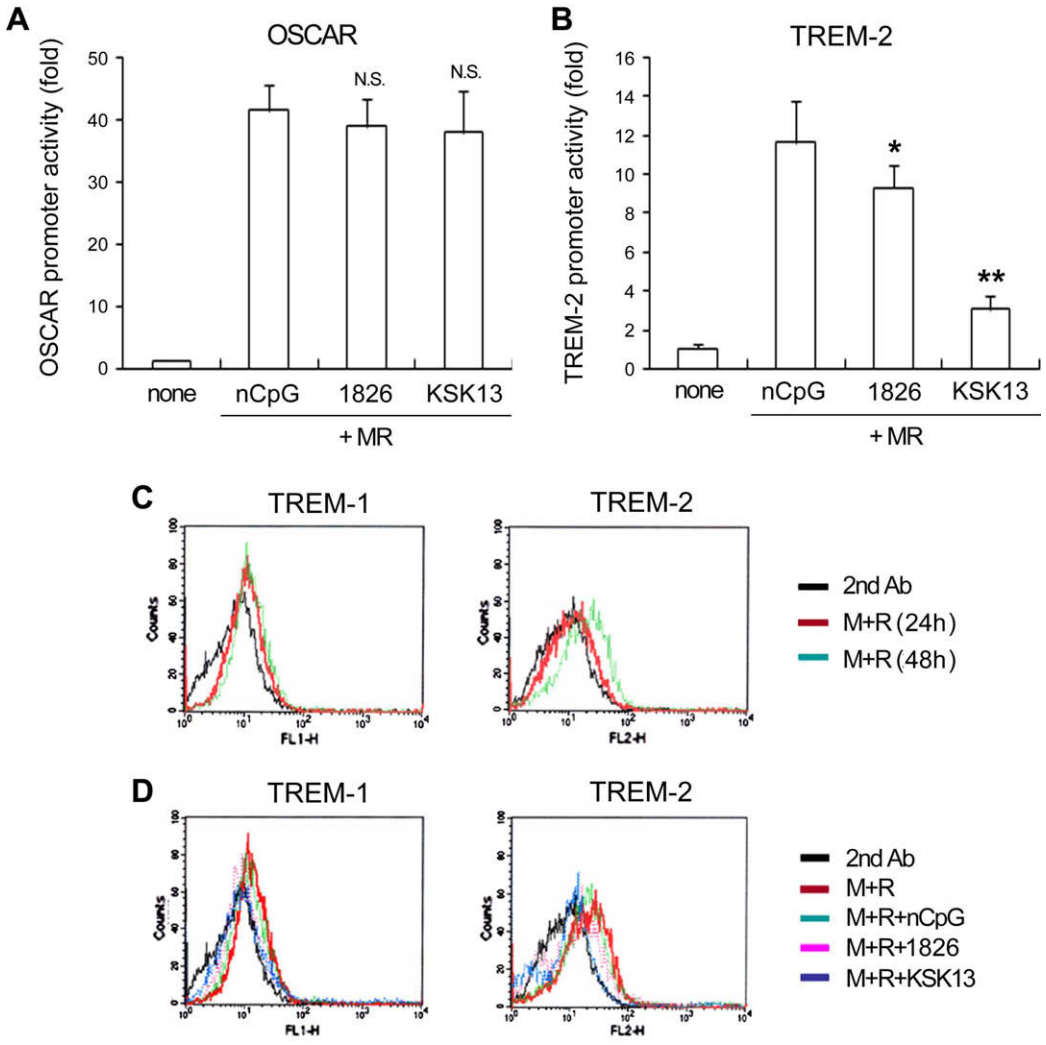


**Fig. 4.** Down-regulation of TREM-2 expression on osteoclast precursors by CpG-KSK13. (A,B) BMMs were transfected with an OSCAR (A) or TREM-2 promoter-dependent reporter plasmid (B). After 24 h, cells were treated with or without nCpG, CpG-1826 (1826) or CpG-KSK13 (KSK13) in the presence of M-CSF (30 ng/ml) plus RANKL (100 ng/ml) for 24 h. Cells were lysed and then subjected to the luciferase assay. N.S, not significant; $^{*}P < 0.05$; $^{**}P < 0.01$ as compared with nCpG-treated group. +MR, M-CSF plus RANKL. (C) Induction of TREM-1 and TREM-2 surface expression by M-CSF plus RANKL. BMMs were cultured with M-CSF (30 ng/ml) and RANKL (100 ng/ml) for 24 h (red solid) or 48 h (green solid). (D) Decreased surface expression of TREM-2 by CpG-KSK13 treatment. BMMs were cultured without (red solid) or with nCpG (green solid), CpG-1826 (1826) (pink dashed) or CpG-KSK13 (KSK13) (blue solid) in the presence of M-CSF (30 ng/ml) and RANKL (100 ng/ml) for 48 h. Cells were stained with fluorescein conjugated anti-TREM-1 (left), anti-TREM-2 (right) or an isotype control antibody (black solid, 2nd Ab) and then subjected to flow cytometry analyses. M + R, 30 ng/ml of M-CSF plus 100 ng/ml of RANKL.

our findings suggest new evidence that TREM-2 expression in OC precursors could be regulated by TLR9 activation, the underlying mechanism for down-regulation of TREM-2 by CpG-KSK13 remains unclear. We cannot exclude the possibility that CpG-KSK13 may influence TREM-2 expression by inhibiting the expression of the M-CSF receptor in OC precursors at early steps of osteoclast differentiation similar to the effects of other CpG-ODNs [14,15]. The molecular cross-talk between these two innate immune receptors, TLR9 and TREM-2, in OC precursors should be further defined.

In conclusion, this study shows that a new CpG-ODN, CpG-KSK13, blocks osteoclastogenesis *in vitro* via TREM-2 down-regulation and NFATc1 inhibition. Our results provide a basis for development of a therapy using CpG-KSK13 targeting of TREM-2 for OC-associated bone diseases.

## Acknowledgments

We thank H. Takayanagi (Tokyo Medical and Dental University, JAPAN) for providing the OSCAR and TREM-2 promoter-dependent reporter plasmids. This work was supported by Grants from the 21C Frontier Functional Proteomics Project (FPR08B1-170) and Science Research Center (R11-2008-023-01001-0) to H.H. Kim, and from the National R&D Program for Cancer Control, Ministry for Health, Welfare and Family affairs, Republic of Korea (0920020) to S.K. Kim. E.J. Chang was supported, in part, by the Korea Research Foundation Grant funded by MEST (KRF-2007-359-E00001).

## References

[1] S.L. Teitelbaum, F.P. Ross, Genetic regulation of osteoclast development and function, Nat. Rev. Genet. 4 (2003) 638–649.
[2] J. Li, I. Sarosi, X.Q. Yan, S. Morony, C. Capparelli, H.L. Tan, S. McCabe, R. Elliott, S. Scully, G. Van, S. Kaufman, S.C. Juan, Y. Sun, J. Tarpley, L. Martin, K. Christensen, J. McCabe, P. Kostenuik, H. Hsu, F. Fletcher, C.R. Dunstan, D.L. Lacey, W.J. Boyle, RANK is the intrinsic hematopoietic cell surface receptor that controls osteoclastogenesis and regulation of bone mass and calcium metabolism, Proc. Natl. Acad. Sci. USA 97 (2000) 1566–1571.
[3] T. Suda, N. Takahashi, N. Udagawa, E. Jimi, M.T. Gillespie, T.J. Martin, Modulation of osteoclast differentiation and function by the new members of the tumor necrosis factor receptor and ligand families, Endocr. Rev. 20 (1999) 345–357.

[4] T. Koga, M. Inui, K. Inoue, S. Kim, A. Suematsu, E. Kobayashi, T. Iwata, H. Ohnishi, T. Matozaki, T. Kodama, T. Taniguchi, H. Takayanagi, T. Takai, Costimulatory signals mediated by the ITAM motif cooperate with RANKL for bone homeostasis, Nature 428 (2004) 758–763.
[5] A. Mócsai, M.B. Humphrey, J.A. Van Ziffle, Y. Hu, A. Burghardt, S.C. Spusta, S. Majumdar, L.L. Lanier, C.A. Lowell, M.C. Nakamura, The immunomodulatory adapter proteins DAP12 and Fc receptor gamma-chain (FcRgamma) regulate development of functional osteoclasts through the Syk tyrosine kinase, Proc. Natl. Acad. Sci. USA 101 (2004) 6158–6163.
[6] M.B. Humphrey, L.L. Lanier, M.C. Nakamura, Role of ITAM-containing adapter proteins and their receptors in the immune system and bone, Immunol. Rev. 208 (2005) 50–65.
[7] H. Huang, E.J. Chang, J. Ryu, Z.H. Lee, Y. Lee, H.H. Kim, Induction of c-Fos and NFATc1 during RANKL-stimulated osteoclast differentiation is mediated by the p38 signaling pathway, Biochem. Biophys. Res. Commun. 351 (2006) 99–105.
[8] G. Elson, I. Dunn-Siegrist, B. Daubeuf, J. Pugin, Contribution of toll-like receptors to the innate immune response to Gram-negative and Gram-positive bacteria, Blood 109 (2007) 1574–1583.
[9] H. Shen, A. Iwasaki, A crucial role for plasmacytoid dendritic cells in antiviral protection by CpG ODN-based vaginal microbicide, J. Clin. Invest. 116 (2006) 2237–2243.
[10] M. Takami, N. Kim, J. Rho, Y. Choi, Stimulation by toll-like receptors inhibits osteoclast differentiation, J. Immunol. 169 (2002) 1516–1523.
[11] S.L. McCoy, S.E. Kurtz, F.A. Hausman, D.R. Trune, R.M. Bennett, S.H. Hefeneider, Activation of RAW264.7 macrophages by bacterial DNA and lipopolysaccharide increases cell surface DNA binding and internalization, J. Biol. Chem. 279 (2004) 17217–17223.
[12] G.J. Weiner, The immunobiology and clinical potential of immunostimulatory CpG oligodeoxynucleotides. J. Leukoc. Biol. 68 (2000) 455–463.
[13] A. Amcheslavsky, H. Hemmi, S. Akira, Z. Bar-Shavit, Differential contribution of osteoclast- and osteoblast-lineage cells to CpG-oligodeoxynucleotide (CpG-ODN) modulation of osteoclastogenesis, J. Bone Miner. Res. 20 (2005) 1692–1699.
[14] W. Zou, H. Schwartz, S. Endres, G. Hartmann, Z. Bar-Shavit, CpG oligonucleotides: novel regulators of osteoclast differentiation, FASEB J. 16 (2002) 274–282.
[15] A. Amcheslavsky, Z. Bar-Shavit, Interleukin (IL)-12 mediates the anti-osteoclastogenic activity of CpG-oligodeoxynucleotides, J. Cell Physiol. 207 (2006) 244–250.
[16] Y. Wittrant, S. Théoleyre, C. Chipoy, M. Padrines, F. Blanchard, D. Heymann, F. Rédini, RANKL/RANK/OPG: new therapeutic targets in bone tumours and associated osteolysis, Biochim. Biophys. Acta 1704 (2004) 49–57.
[17] A. Amcheslavsky, W. Zou, Z. Bar-Shavit, Toll-like receptor 9 regulates tumor necrosis factor-alpha expression by different mechanisms. Implications for osteoclastogenesis, J. Biol. Chem. 279 (2004) 54039–54045.
[18] W. Zou, A. Amcheslavsky, Z. Bar-Shavit, CpG oligodeoxynucleotides modulate the osteoclastogenic activity of osteoblasts via toll-like receptor 9, J. Biol. Chem. 278 (2003) 16732–17640.
[19] S. Bauer, C.J. Kirschning, H. Hacker, V. Redecke, S. Hausmann, S. Akira, H. Wagner, G.B. Lipford, Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition, Proc. Natl. Acad. Sci. USA 98 (2001) 9237–9242.
[20] H.C. Cho, B.H. Kim, K. Kim, J.Y. Park, J.H. Chang, S.K. Kim, Cancer immunotherapeutic effects of novel CpG-ODN in murine tumor model, Int. Immunophamacol. 8 (2008) 1401–1407.
[21] S.J. Park, H.C. Cho, K.S. Bae, S.K. Kim, Immunoadjuvanticity of novel CpG-ODN, Mol. Cell. Toxicol. 3 (2007) 46–52.
[22] E.J. Chang, H.J. Kim, J. Ha, H.J. Kim, J. Ryu, K.H. Park, U.H. Kim, Z.H. Lee, H.M. Kim, D.E. Fisher, H.H. Kim, Hyaluronan inhibits osteoclast differentiation via toll-like receptor 4, J. Cell Sci. 120 (2007) 166–176.
[23] T.L. Roberts, M.J. Sweet, D.A. Hume, K.J. Stacey, Cutting edge: species-specific TLR9-mediated recognition of CpG and non-CpG phosphorothioate-modified oligonucleotides, J. Immunol. 174 (2005) 605–608.
[24] S.G. Mohamed, E. Sugiyama, K. Shinoda, H. Taki, H. Hounoki, H.O. Abdel-Aziz, M. Maruyama, M. Kobayashi, H. Ogawa, T. Miyahara, Interleukin-10 inhibits RANKL-mediated expression of NFATc1 in part via suppression of c-Fos and c-Jun in RAW264.7 cells and mouse bone marrow cells, Blood 41 (2007) 592–602.
[25] Y. Kim, K. Sato, M. Asagiri, I. Morita, K. Soma, H. Takayanagi, Contribution of nuclear factor of activated T cells c1 to the transcriptional control of immunoreceptor osteoclast-associated receptor but not triggering receptor expressed by myeloid cells-2 during osteoclastogenesis, J. Biol. Chem. 280 (2005) 32905–32913.
[26] M.B. Humphrey, M.R. Daws, S.C. Spusta, E.C. Niemi, J.A. Torchia, L.L. Lanier, W.E. Seaman, M.C. Nakamura, TREM2, a DAP12-associated receptor, regulates osteoclast differentiation and function, J. Bone Miner. Res. 21 (2006) 237–245.
[27] M. Cella, C. Buonsanti, C. Strader, T. Kondo, A. Salmaggi, M. Colonna, Impaired differentiation of osteoclasts in TREM-2-deficient individuals, J. Exp. Med. 198 (2003) 645–651.