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# TAK1 is a pivotal therapeutic target for tumor progression and bone destruction in myeloma

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## ABSTRACT

Along with tumor progression, the bone marrow microenvironment is skewed in multiple myeloma (MM), which underlies the unique pathophysiology of MM and confers aggressiveness and drug resistance in MM cells. TGF-β-activated kinase-1 (TAK1) mediates a wide range of intracellular signaling pathways. We demonstrate here that TAK1 is constitutively overexpressed and phosphorylated in MM cells, and that TAK1 inhibition suppresses the activation of NF-κB, p38MAPK, ERK and STAT3 in order to decrease the expression of critical mediators for MM growth and survival, including PIM2, MYC, Mcl-1, IRF4, and Sp1, along with a substantial reduction in the angiogenic factor VEGF in MM cells. Intriguingly, TAK1 phosphorylation was also induced along with upregulation of vascular cell adhesion molecule-1 (VCAM-1) in bone marrow stromal cells (BMSC) in cocultures with MM cells, which facilitated MM cell-BMSC adhesion while inducing IL-6 production and receptor activator of nuclear factor κ-B ligand (RANKL) expression by BMSC. TAK1 inhibition effectively impaired MM cell adhesion to BMSC to disrupt the support of MM cell growth and survival by BMSC. Furthermore, TAK1 inhibition suppressed osteoclastogenesis enhanced by RANKL in cocultures of bone marrow cells with MM cells, and restored osteoblastic differentiation suppressed by MM cells or inhibitory factors for osteoblastogenesis overproduced in MM. Finally, treatment with the TAK1 inhibitor LLZ1640-2 markedly suppressed MM tumor growth and prevented bone destruction and loss in mouse MM models. Therefore, TAK1 inhibition may be a promising therapeutic option targeting not only MM cells but also the skewed bone marrow microenvironment in MM.

## Introduction

Multiple myeloma (MM) has a unique propensity to almost exclusively develop in the bone marrow and generate devastating bone destruction. MM cells enhance osteoclast (OC) formation and activity, and suppress osteoblastic differentiation from bone marrow stromal cells (BMSC), leading to extensive bone destruction with rapid development of osteolytic lesions.[1,2] Angiogenesis is also enhanced through these cellular interactions.[3,4] The types of cells surrounding MM cells create a cellular microenvironment suitable for MM cell growth and survival to confer drug resistance, which can be termed the "MM niche".

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In order to improve therapeutic efficacy, we need to disrupt the MM niche that confers drug resistance. Therefore, we looked for novel molecules upregulated in the MM niche to be targeted, and found that proviral integrations of Moloney virus 2 kinase (PIM2) is constitutively overexpressed as an anti-apoptotic mediator in MM cells.[5] PIM2 expression has been demonstrated to be higher in hematologic malignancies than solid cancers or their normal tissue counterparts, and highest in MM.[6,7] Importantly, PIM2 can be further upregulated in MM cells in cocultures with BMSC as well as OC.[5]

Although multiple soluble inhibitors for osteoblastogenesis have been reported to be overproduced in MM, including IL-3,[8] IL-7,[9] TNF-α,[10] TGF-β,[11] and activin A,[12] PIM2 was found to be upregulated in BMSC by these inhibitory factors acting as a common intracellular mediator to suppress their osteoblastogenesis.[13] Moreover, we subsequently reported that PIM2 is induced in osteoclastic lineage cells by receptor activator of nuclear factor κ-B ligand (RANKL) to act as a critical mediator of RANKL-induced osteoclastogenesis in MM.[14] Therefore, PIM2 appears to play a versatile role in tumor progression and bone destruction and bone loss in MM, and is regarded as an important therapeutic target.

TGF-β-activated kinase 1 (TAK1) is a member of the mitogen-activated protein kinase kinase (MAP3K) family, also known as MAP3K7.[15,16] It was originally identified as a key kinase in transducing TGF-β signaling down to p38 mitogen-activated protein kinase (MAPK) and c-Jun and N-terminal kinase (JNK).[15] Subsequently, TAK1 has been demonstrated to be associated with the activation of a wide range of intracellular signaling pathways important for various cellular functions, including the activation of nuclear factor-κB (NF-κB) and extracellular signal-regulated kinase (ERK).[15] Therefore, TAK1 appears to be a gate keeper to facilitate the multiple important intracellular signaling pathways. Therapeutic efficacy of TAK1 inhibition has been preclinically demonstrated in different types of cancers, including mantle cell lymphoma,[17] breast cancer,[18] and colon cancer.[19]

We demonstrate here that TAK1 is constitutively overexpressed and phosphorylated in MM cells and that TAK1 acts as an upstream regulator responsible for multiple signaling pathways critical for MM growth and survival.[1] TAK1 phosphorylation is also induced in BMSC in cocultures with MM cells, which facilitates MM cell adhesion to BMSC between very late antigen-4 (VLA-4) and vascular cell adhesion molecule-1 (VCAM-1), thereby inducing IL-6 production and RANKL expression by BMSC. Importantly, TAK1 inhibition was able to effectively induce MM cell death, and alleviate bone destruction though suppression of osteoclastogenesis and restoration of osteoblastogenesis. Therefore, TAK1 appears to be a pivotal therapeutic target in MM to disrupt the key signal transduction pathways responsible for tumor progression and bone destruction.

## Methods

### Ethics

All procedures involving human samples from healthy donors and patients were performed with written informed consent in accordance with the Declaration of Helsinki and a protocol approved by the Institutional Review Board for human protection at the University of Tokushima (Permission number: 240). All animal experiments were conducted under the regulation and permission of the Animal Care and Use Committee of Tokushima University, Tokushima, Japan (toku-dobutsu 13094).

### Reagents

Reagents used in this manuscript are described in the *Online Supplementary Appendix*.

### Cells and cell culture

Details of the cells and cell culture procedures are available in the *Online Supplementary Appendix*.

### Western blotting

Cells were collected and lysed in RIPA lysis buffer (Santa Cruz, Dallas, TX, USA). For cytosolic and nuclear preparation, cells were lysed in NE-PER extraction reagent (Thermo Fisher Scientific, Waltham, MA) in accordance with the manufacturer's protocol. Western blot analysis was done with equal protein amounts of cell lysate, as described previously.[13]

### Cell viability

Cell viability was determined using the Cell Counting Kit-8 assay (Dojindo, Kumamoto, Japan) in accordance with the manufacturer's instructions. The absorbance of each well was measured at 450-655 nm using an iMark microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). In order to assess apoptotic cells, cells were stained with an annexinV-FITC and propidium iodide labeling kit (MEBCYTO Apoptosis Kit; MBL, Nagano, Japan) in accordance with the manufacturer's instruction, and analyzed by flow cytometry.

### Small interfering RNA transfection

Small interfering RNA (siRNA) transduction was performed as described previously.[5,20] Human and mouse *TAK1* siRNA were purchased from Santa Cruz. Human *TAK1* siRNA was transfected into MM cells using a Human Nucleofector Kit (Lonza, Basel, Switzerland). Mouse *TAK1* siRNA was transfected into mouse BMSC or RAW264.7 cells using siRNA Transfection Reagent (Santa Cruz) in accordance with the manufacturer's protocol.

### Real-time reverse transcription polymerase chain reaction

RNA isolation and quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) were performed as described previously.[20] The following primer sequences were used: human *RANKL* F: TCGTTGGATCACAGCACATCA and R: TATGGGAACCAGATGGGATGTC, human *IL-6* F: TCTGAGGCTCATTCTGCCCTCGAGC and R: AACTGGACCGAAGGCGCTTGTGGA, human *GAPDH*, used as an endogenous control to normalize each sample, F: TGTCTTCACCACCATGGAGAAGG and R: GTGGATGCAGGGATGATGTTCTG

### Adhesion assays

Adhesion assays were performed as described previously.[21] Human BMSC ($2\times10^4$ cells/well) were expanded in 96-well culture plates. MM cells were labeled with BCECF-AM (Dojindo) for 2 hours at 37°C and 5% $CO_2$ as described previously.[22] BMSC were washed, and the fluorescence-labeled MM cells were added onto the BMSC and incubated for 4 hours. Nonadherent BCECF-AM-labeled cells were removed by gently pipetting four times. Adherent cells were quantitated in a fluorescence multi-well plate reader (Infinite® 200 PRO, TECAN, Männedorf. Switzerland).

### Multiple myeloma animal model and histological analyses

Multiple myeloma (MM) mouse models were prepared by intra-tibial inoculation of mouse *luciferase*-transfected 5TGM1 MM cells (a gift from Dr. Gregory R. Mundy [Vanderbilt Center for Bone Biology, Vanderbilt University, Nashville, TN, USA]) into ICR nu/nu mice (CLEA Japan) at 4–6 weeks old as described previously.[13,14] The assessment of tumor growth and bone volume, and bone histomorphometric and immunohistochemical analyses are described in the *Online Supplementary Appendix*.

### Statistical analysis

Statistical analysis was performed using Student's *t*-test or one-way analysis of variance (ANOVA). $P<0.05$ was considered as a significant difference. All statistics were performed using the Statistical Package for Social Sciences (SPSS 13.0 for Windows; Chicago, IL, USA).

## Results

### TAK1 is activated to mediate growth and survival of multiple myeloma cells

We first examined the expression and activation status of TAK1 in MM cells. TAK1 is highly overexpressed and phosphorylated in MM cell lines and primary MM cells from patients, whereas normal peripheral blood mononuclear cells (PMBC) only weakly expressed TAK1 (Figure 1A). IRF4 and MYC have been regarded as master regulators for MM cell survival and function, which MM cells are addicted to, and their reduction is a major mechanism for the anti-MM activity of immunomodulatory drugs lenalidomide and pomalidomide.[23,24] Notably, the TAK1 inhibitor LLZ dose-dependently reduced MYC and IRF4 in parallel with the reduction in PIM2 expression and the phosphorylation of the PIM2 substrate 4E-BP1 as well as the anti-apoptotic factor Mcl-1 in MM cells (Figure 1B, left). We and others reported that transcription factor Sp1 is constitutively overexpressed in MM cells, which can serve as an important therapeutic target for MM.[25-27] TAK1 inhibition also substantially reduced Sp1 (Figure 1B, left). The effects of TAK1 inhibition were confirmed with *TAK1* knockdown using siRNA (Figure 1B, right).

TAK1 inhibition with LLZ dose-dependently induced cell death in all MM cell lines tested (Figure. 1C, left), and *TAK1* knockdown also reduced the viability of MM cells (Figure 1C, right). The induction of apoptosis was confirmed using annexinV and propidium iodide dual staining (Figure 1D) with activation of caspase-8, caspase-9, and caspase-3 (Figure 1E) in MM cells, indicating activation of the extrinsic as well as the intrinsic caspase-mediated apoptotic pathways. LLZ at the concentrations up to 10 μM did not apparently impair the viability of normal PBMC (*Online Supplementary Figure S1*). Therefore, TAK1 appears to be a good therapeutic target to effectively induce cell death in MM cells through suppression of PIM2 plus other pro-survival mediators.

### TAK1 inhibition is able to abolish IL-6 and TNF-α-induced signaling in multiple myeloma cells

IL-6 and TNF-α are predominant paracrine factors overproduced in the bone marrow microenvironment in MM, and these elicit the signaling pathways responsible for MM cell growth and survival.[28] As we demonstrated previously,[5] PIM2 was substantially upregulated in MM cell lines in cocultures with BMSC as well as in the presence of IL-6 or TNF-α (Figure 2A). However, treatment with LLZ was able to abolish PIM2 upregulation in MM cells by BMSC as well as IL-6 or TNF-α. We further examined the effects of TAK1 inhibition on the signaling pathways in MM cells activated by IL-6 and TNF-α. After starving without serum, phosphorylation of TAK1 was reduced in RPMI8226 cells, but IL-6 (Figure 2B) and TNF-α (Figure 2C) promptly induced the phosphorylation of TAK1 and activation of their corresponding downstream signaling molecules in serum-depleted media. Treatment with LLZ abolished IL-6-induced phosphorylation of STAT3 (Figure 2B), and TNF-α-induced phosphorylation and degradation of IκBα and phosphorylation of p38MAPK and ERK (Figure 2C) in the MM cells. Analyses of nuclear extracts from MM cells treated with TNF-α showed the nuclear accumulation of p65; however, treatment with LLZ reduced the p65 content in the nuclear extracts in the presence of TNF-α (Figure 2D). Consistent with the notion that PIM2 is transcriptionally upregulated in MM cells by the NF-κB signaling pathway,[5] the PIM inhibitor SMI-16a showed only marginal effects on the nuclear accumulation of p65 induced by TNF-α (Figure 2D). Of note, the TAK1 inhibitor LLZ as well reduced MM cell viability in cocultures with BMSC (Figure 2E). *TAK1* knockdown in MM cells mostly reduced their viability even in cocultures with BMSC (Figure 2F). Also, *TAK1* knockdown in BMSC partially but significantly reduced their supportive activity for MM cell growth (Figure 2G). Therefore, TAK1 activation in both MM cells and BMSC is suggested to play an important role in MM cell growth and survival in cocultures with BMSC. Furthermore, although cytotoxic effects of doxorubicin on MM cells were blunted in cocultures with BMSC, TAK1 inhibition with LLZ was able to resume MM cell death by doxorubicin even in the presence of BMSC (*Online Supplementary Figure S2*). These results suggested that TAK1 inhibition impairs MM cell growth and survival supported by the bone marrow microenvironment.

### TAK1 inhibition impairs multiple myeloma cell adhesion to bone marrow stromal cells

MM cell adhesion to BMSC through the interaction between VLA-4 and its corresponding ligand, VCAM-1, is among the predominant mechanisms for cell adhesion-mediated drug resistance (CAM-DR) in MM,[22,29] while enhancing the production of IL-6[30] and RANKL, an critical osteoclastogenic factor in MM.[31] In addition to TAK1 activation in MM cells, TAK1 was found to be phosphorylated along with PIM2 upregulation in BMSC, when cocultured with MM cells (Figure 3A). Furthermore, VCAM-1 expression was substantially upregulated in BMSC after coculturing with MM cells, which was abolished by TAK1 inhibition with LLZ (Figure 3B). TNF-α is known as a potent inducer of VCAM-1 in BMSC through activation of the NF-κB signaling pathway.[32,33] Treatment with LLZ as well as *TAK1* knockdown by siRNA abrogated the upregulation of VCAM-1 expression in BMSC by TNF-α (Figures 3B and 3C). Treatment with TNF-α promptly phosphorylated TAK1 and degraded IκBα, and induced the phosphorylation of p38MAPK and ERK in BMSC (Figure 3D). However, TAK1 inhibition with LLZ as well as *TAK1* knockdown abolished the degradation of IκBα and reduced the phosphorylation of p38MAPK and ERK in the presence of TNF-α, indicating efficacious suppression

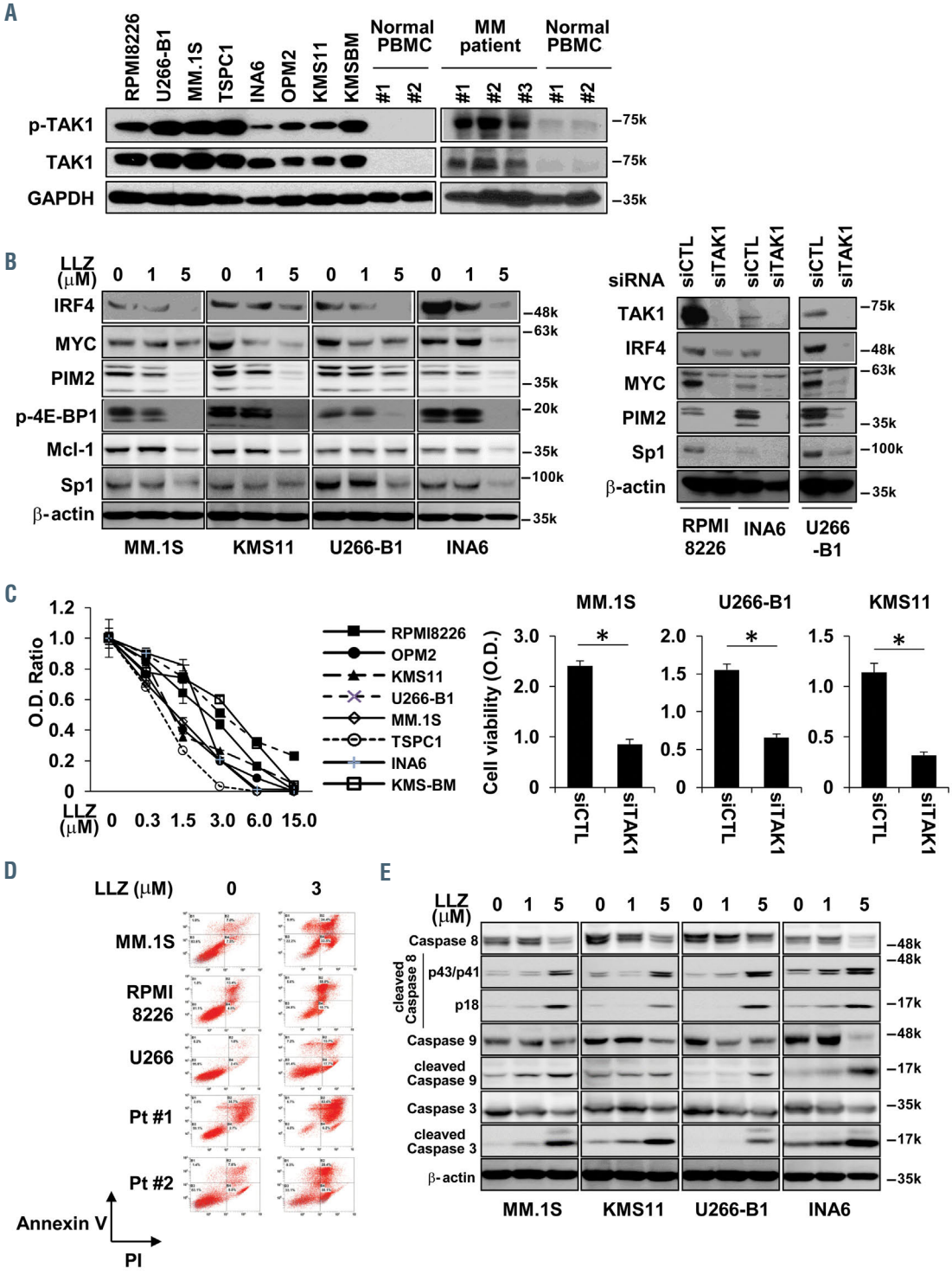


**Figure 1. TAK1 is activated to mediate growth and survival of multiple myeloma cells.** (A) TAK1 expression and phosphorylation in multiple myeloma (MM) cell lines. TAK1 expression and phosphorylation were examined by western blotting analysis in various MM cell lines and primary MM cells as well as normal peripheral blood mononuclear cells (PBMC). Glyceraldehyde 3-phosphate dehydrogenase (GADPH) was used as a loading control. (B) The indicated MM cell lines were cultured in the absence or presence of LLZ at 1 or 5 μM for 2 days (left). The cells were transduced with scrambled (siCTL) or human *TAK1* small interfering RNA (siRNA) (siTAK1) and cultured for 13 hours (right). Cell lysates were then collected, and protein levels of the indicated molecules were analyzed by western blotting analysis. β-actin was used as a loading control. (C) MM cell lines as indicated were incubated in triplicate in the presence of the indicated concentrations of TAK1 inhibitor LLZ1640-2 (LLZ) for 48 hours (left). The indicated MM cell lines transduced with scrambled (siCTL) or human *TAK1* siRNA (siTAK1) were cultured in triplicate for 24 hours (right). Cell viability was measured using a WST-8 assay. Data are expressed as means ± standard deviation. (D) The indicated MM cell lines and CD138-positive cells isolated from MM patients (Pt #1 and Pt #2) were cultured in the presence or absence of LLZ at 3 μM for 24 hours. The induction of apoptosis was analyzed using annexinV and propidium iodide (PI) dual staining. (E) The indicated MM cell lines were cultured alone or in the presence of LLZ at 1 or 5 μM for 2 days, and cell lysates were then collected. Caspase-mediated apoptotic pathways were analyzed using western blotting analysis. β-actin was used as a loading control.

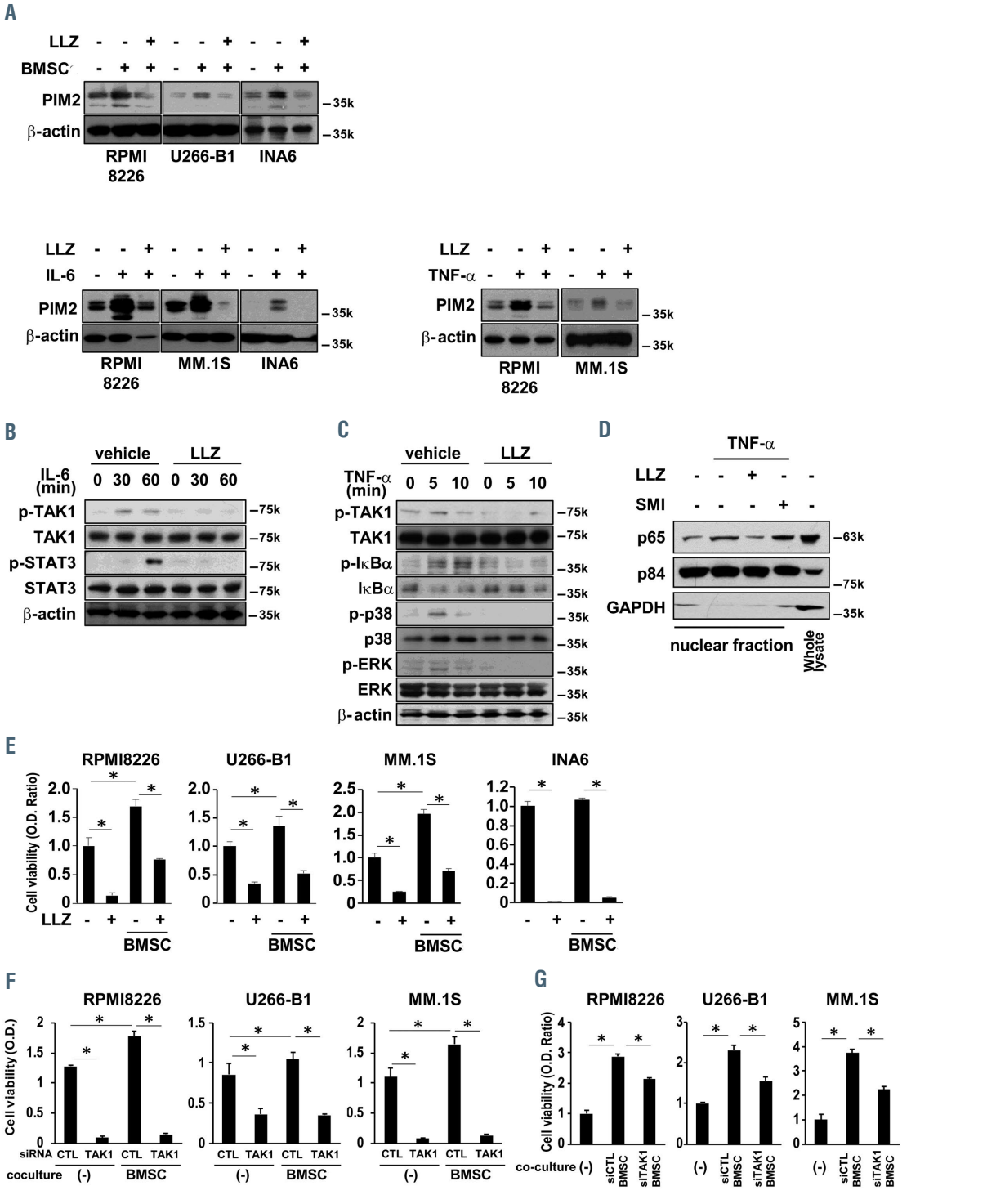


**Figure 2. TAK1 inhibition abolishes IL-6- and TNF-α-induced signaling in multiple myeloma cells.** (A) Human bone marrow stromal cells (BMSC) were expanded in 24-well culture plates. The indicated multiple myeloma (MM) cell lines were cultured alone or cocultured with BMSC (upper), or cultured in the presence or absence of IL-6 (lower left) or TNF-α (lower right) for 24 hours (h), and cell lysates were then collected. PIM2 expression was analyzed using western blotting. β-actin was blotted as a loading control. (B, C) RPMI8226 cells were cultured in α-MEM with 1% FBS for 12 h for serum starvation. The starved cells were then cultured in α-MEM with 1% fetal bovine serum (FBS) with or without LLZ1640-2 (LLZ) at 3 μM. Three hours later, IL-6 (b) or TNF-α (c) at 10 ng/mL was added. After the indicated time periods, cell lysates were collected. The expression of phosphorylated TAK1 (p-TAK1), TAK1, phosphorylated STAT3 (p-STAT3), STAT3, phosphorylated IκBα (p-IκBα), IκBα, phosphorylated p38MAPK (p-p38), p38MAPK (p38), phosphorylated ERK (p-ERK), and ERK were detected using western blotting. β-actin was used as a loading control. (D) RPMI8226 cells after starvation were cultured in α-MEM with 1% FBS with or without LLZ (5 μM) or the PIM inhibitor SMI-16a (50 μM). Three hours later, TNF-α at 10 ng/mL was added as indicated. After incubating for 15 minutes, whole lysates and nuclear fractions were extracted. The protein levels of p65 were analyzed using western blotting. Glyceraldehyde 3-phosphate dehydrogenase (GADPH) and p84 were used as protein loading controls for cytoplasmic and nuclear proteins, respectively. (E) The indicated MM cell lines were cultured alone or cocultured with human BMSC expanded in 24-well culture plates for 24 hours in the presence or absence of LLZ at 5 μM. MM cells were then collected, and their viability was analyzed using a WST8 assay. (F) The indicated MM cell lines were transduced with scrambled (siCTL) or *TAK1* siRNA (siTAK1), cultured alone or cocultured with human BMSC expanded in 24-well culture plates for 24 hours. MM cells were then collected, and their viability was analyzed using a WST8 assay. (G) Indicated MM cells were cocultured for 24 hours with or without human BMSC which were transduced with scrambled (siCTL BMSC) or *TAK1* siRNA (siTAK1 BMSC). MM cells were then collected, and their viability was analyzed using a WST8 assay.

of TNF-α signaling in BMSC by TAK1 inhibition. VLA-4, α4 β1 integrin, is constitutively overexpressed in MM cells. We found that the TAK1 inhibition with LLZ was able to reduce the expression of β1 integrin in MM cells (*Online Supplementary Figure S3*), indicating the contribution of TAK1 to β1 integrin expression in MM cells. Consistently, treatment with LLZ suppressed MM cell adhesion to BMSC (Figures 3E; *Online Supplementary Figure S4*), although LLZ did not impair the viability of BMSC (*Online Supplementary Figure S5*).

BMSC are regarded as a major source of IL-6, a growth and survival factor for MM cells, and the critical osteoclastogenic factor RANKL. Consistent with the previous observations,[30,31] cocultures with MM cells potently augmented IL-6 (Figures 3F) and RANKL (Figures 3G) mRNA expression in BMSC. However, treatment with LLZ suppressed the upregulation of these factors in BMSC in cocultures with MM cells. These data demonstrate that TAK1 inhibition is able to efficaciously suppress MM cell adhesion to BMSC and thereby abolish the upregulation of IL-6 and RANKL in BMSC, which may alleviate MM tumor progression in the bone marrow and bone destruction.

### TAK1 inhibition suppresses osteoclastogenesis enhanced by RANKL and multiple myeloma cells

Consistent with previous observations,[20,34] RANKL induced the phosphorylation of TAK1 in parallel with the degradation of IκBα and phosphorylation of p38MAPK and ERK (Figure 4A), and nuclear localization of the NF-κB subunit p65 (Figure 4B) in RAW264.7 preosteoclastic cells. However, treatment with LLZ abolished all of these RANKL-mediated changes, indicating critical involvement of TAK1 in RANKL-induced activation of the NF-κB and MAPK pathways. RANKL induced the expression of NFATc1 and c-fos, critical transcription factors for osteoclastogenesis (Figure 4C), and the formation of TRAP-positive multinucleated cells, namely OC, in RAW264.7 cells (Figures 4D); however, treatment with LLZ dose-dependently suppressed the RANKL-induced expression of NFATc1 and c-fos, and OC formation. *TAK1* knockdown by siRNA also abolished the induction of NFATc1 and c-fos expression (Figure 4E) and osteoclastogenesis (Figure 4F) by RANKL. Furthermore, MM cells potently induced TRAP-positive multinucleated OC formation from bone marrow cells; however, treatment with LLZ suppressed OC formation (Figure 4G). These results demonstrate that TAK1 inhibition is able to suppress osteoclastogenesis enhanced by MM cells.

### TAK1 inhibition restores osteoblastogenesis suppressed by multiple myeloma cells as well as major inhibitors for osteoblastogenesis in multiple myeloma

In contrast to the enhanced osteoclastogenesis, osteoblastogenesis or bone formation is suppressed in MM. Conditioned media (CM) from MM cell lines as well as inhibitory factors for osteoblastogenesis overproduced in MM, including IL-3, IL-7, TNF-α, TGF-β, and activin A,[8-12] induced the phosphorylation of TAK1 (Figure 5A) and suppressed mineralized nodule formation (Figure 5B) in MC3T3-E1 preosteoblastic cells. However, treatment with the TAK1 inhibitor LLZ restored mineralized nodule formation (Figure 5B). Osterix is an essential transcription factor for osteoblastogenesis, known as a downstream target of BMP-2. The upregulation of Osterix by BMP-2 was reduced in MC3T3-E1 cells in the presence of CM from MM cell lines or TNF-α; however, treatment with LLZ restored the upregulation of Osterix by BMP-2 (Figure 5C).

TGF-β inhibits the terminal stage of OB differentiation or bone mineralization, whereas BMP-2 is a stimulator for osteoblastogenesis.[11,35-38] We and others demonstrated that TGF-β plays a significant role in bone destruction in MM, and that the inhibition of the TGF-β signaling restored bone formation in MM animal models.[11,39-41] Treatment with TGF-β induced the phosphorylation of Smad2 and Smad3 in MC3T3-E1 cells (Figure 5D). However, TAK1 inhibition with LLZ as well as *TAK1* knockdown by siRNA abolished the phosphorylation of these factors. TGF-β has been shown to counteract the BMP-2 signaling to suppress the terminal differentiation of OB in part through the upregulation of Smad6, an inhibitory regulator for BMP-2 signaling.[42] Treatment with LLZ for 24 hours dose-dependently reduced Smad6 protein levels in MC3T3-E1 cells (Figure 5E, upper). Moreover, LLZ inhibited TGF-β-induced upregulation of Smad6 in MC3T3-E1 cells (Figure 5E, lower). In contrast, treatment with LLZ as well as *TAK1* knockdown by siRNA enhanced the phosphorylation of Smad1/5 in MC3T3-E1 cells by BMP-2 (Figure 5F). These results collectively suggest that TAK1 inhibition may resume osteoblastogenesis suppressed in MM.

### TAK1 inhibition suppresses vascular endothelial growth factor secretion by multiple myeloma cells

Angiogenesis also plays an important role in the pathogenesis and progression of MM. Vascular endothelial growth factor (VEGF) appears to be the most critical angiogenic factor in MM.[43,44] VEGF has been demonstrated to be overproduced downstream of the signaling mediator ERK in MM cells.[45] As expected, TAK1 inhibition with LLZ as well as *TAK1* knockdown with siRNA substantially reduced VEGF production by MM cells (*Online Supplementary Figure S6*). These results suggested that TAK1 inhibition can impair angiogenesis in MM to retard MM progression.

### TAK1 inhibition suppresses multiple myeloma tumor progression and prevents bone destruction *in vivo*

We next examined the *in vivo* effects of the TAK1 inhibitor LLZ using MM mouse models by intratibial inoculation of mouse 5TGM1 MM cells. Mice were treated with LLZ every other day for 2 weeks from day 6, the day on which 5TGM1 MM cell-derived IgG2b levels started to increase in mouse sera. Vehicle-treated mice showed at day 21 large tumor masses around the tibiae where MM cells were inoculated (Figure 6A), and a progressive increase in serum IgG2b levels over time (Figure 6B). Bone destruction in the tibiae was observed at day 21 in plain X-ray as well as μ-computed tomography (μ-CT) images (Figure 6C). Treatment with LLZ substantially suppressed tumor sizes (Figure 6A) with almost no increase in serum IgG2b (Figure 6B), and prevented bone destruction of the tibiae (Figure 6C). Cathepsin K-expressing OC increased in number on the surface of bone in 5TGM1-inoculated tibiae; however, treatment with LLZ reduced the OC numbers (Figure 6D). These results demonstrate that TAK1 inhibition is able to suppress MM tumor growth while preventing bone destruction *in vivo*.

In order to further clarify the effects of TAK1 inhibition

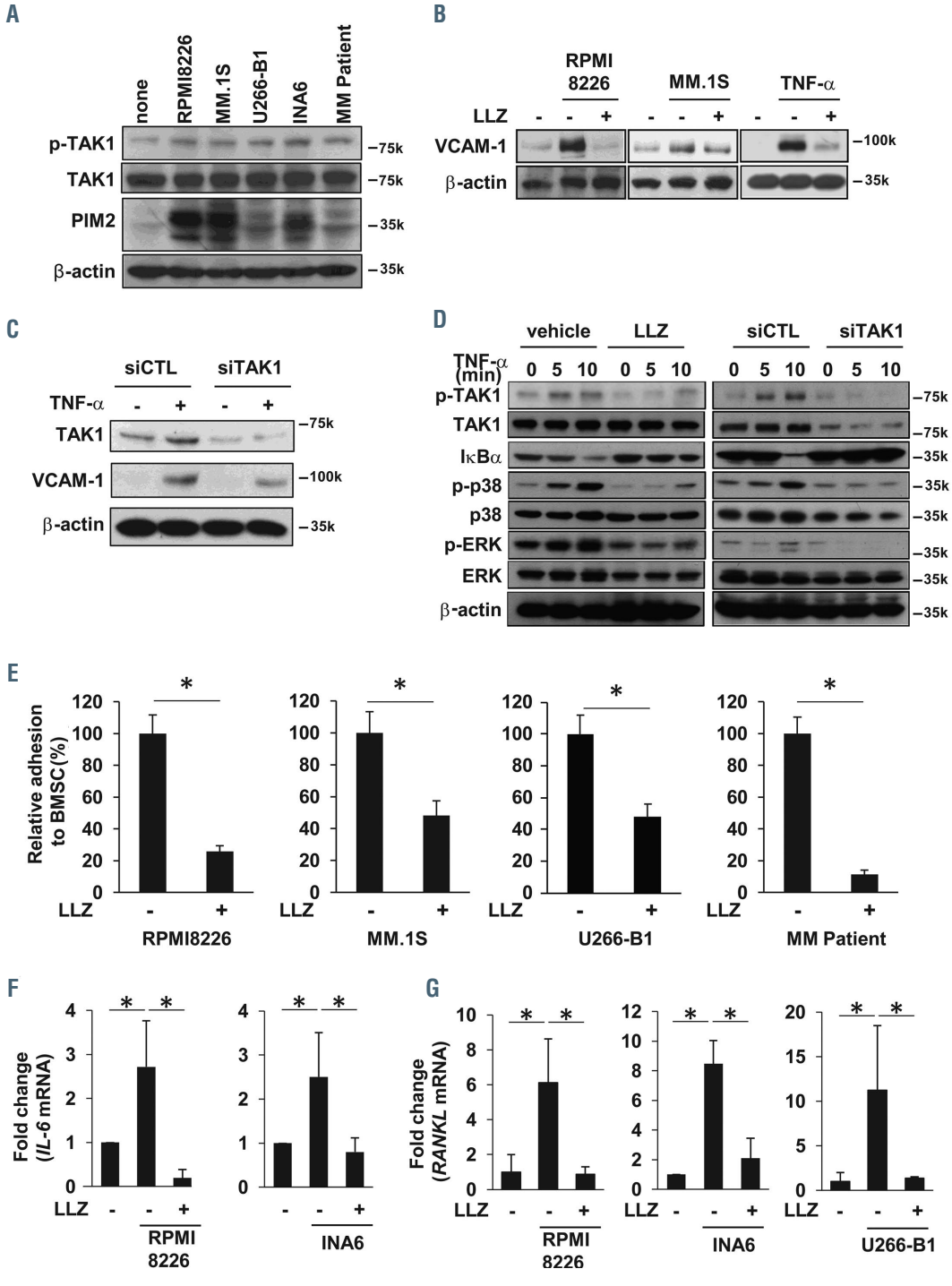


**Figure 3. TAK1 inhibition suppresses VCAM-1 expression in bone marrow stromal cells and their adhesion to multiple myeloma cells.** (A) Human bone marrow stromal cells (BMSC) were expanded in 6-well culture plates. The BMSC were cocultured with the indicated multiple myeloma (MM) cell lines for 24 hours. After washing out MM cells, cell lysates were collected from the BMSC. The indicated protein levels were examined using western blotting. (B) Human BMSC were cultured alone or cocultured with the indicated MM cell lines, or cultured with TNF-α at 10 ng/mL in the presence or absence of LLZ (5 μM) for 2 days. Cell lysates were collected from BMSC, and VCAM-1 expression was analyzed using western blotting. (C) BMSC were transduced with scrambled (siCTL) or *TAK1* small interfering RNA (siRNA) (siTAK1), and then cultured for 2 days with or without TNF-α at 10 ng/mL. Cell lysates were collected, and VCAM-1 expression was analyzed using western blotting. (D) BMSC cells were starved in α-MEM with 1% fetal bovine serum (FBS) for 12 hours. The cells were then cultured in α-MEM with 1% FBS with or without LLZ at 5 μM for 3 hours (left), or transduced with scrambled (siCTL) or *TAK1* siRNA (siTAK1) (right). TNF-α at 10 ng/mL was added and cells lysates were harvested after the indicated time periods. The indicated protein levels were analyzed using western blotting. (E) Human BMSC were treated with LLZ (5 μM) for 1 day, and the indicated MM cells were then added in quadruplicate at $10^5$ cells/well, and incubated for 4 hours. By gentle pipetting, non-adherent MM cells were removed, and adherent MM cells were quantitated in a fluorescence multi-well plate reader. Data represent the means ± standard deviation (SD) (n=4). **P*<0.05, by ANOVA. (F, G) Human BMSC prepared in 6-well culture plates were cultured in triplicate alone or cocultured with MM cells as indicated in the presence or absence of LLZ (5 μM) for 1 day. After washing out MM cells, total RNA was isolated from the BMSC. *IL-6* (f) and *RANKL* (g) mRNA expression in the BMSC was determined using quantitative reverse transcription polymerase chain reaction. Data represent the means ± SD (n=3). **P*<0.05, by ANOVA.

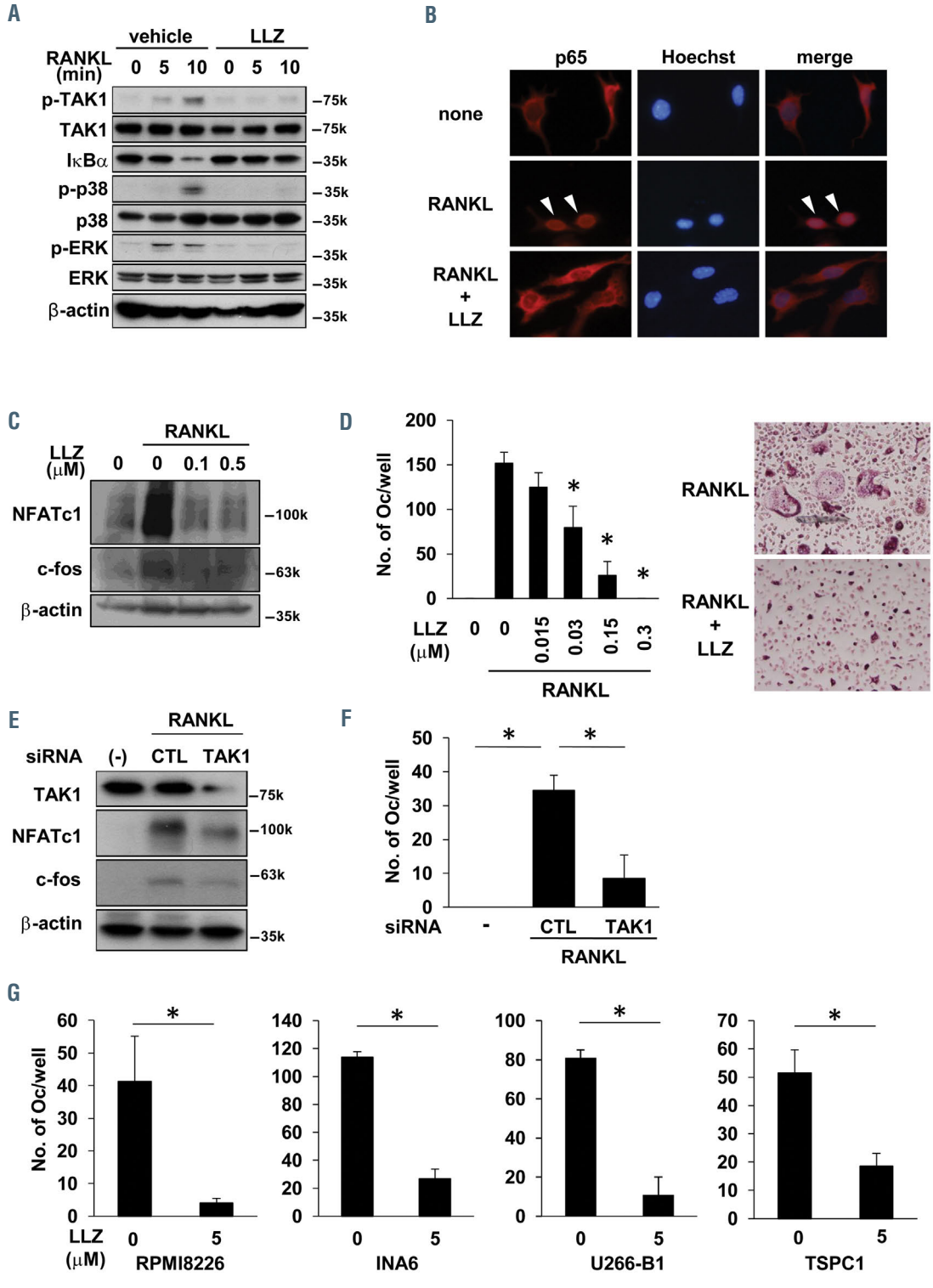


**Figure 4. TAK1 inhibition suppresses osteoclastogenesis enhanced by RANKL and multiple myeloma cells.** (A) RAW264.7 cells were starved in α-MEM with 1% fetal bovine serum (FBS) for 12 hours, and then treated with or without LLZ at 1 μM for 3 hours, followed by the addition of RANKL (20 ng/mL) for the indicated time periods. Phosphorylated TAK1 (p-TAK1), TAK1, IκBα, phosphorylated p38MAPK (p-p38MAPK), p38MAPK, phosphorylated ERK (p-ERK), and ERK protein levels were analyzed using western blotting. β-actin was used as a loading control. (B) In order to observe p65 localization, the cells were fixed and stained with anti-p65 antibody (red), and their nuclei were stained with Hoechst 33342 (blue). (C) RAW264.7 cells were treated with or without RANKL at 25 ng/mL for 1 day. LLZ was added as indicated. Protein levels of NFATc1 and c-fos were determined using western blotting. (D) Primary mouse bone marrow cells were cultured in quadruplicate with or without RANKL (25 ng/mL) for 4 days. LLZ was added at the indicated concentrations. Then, the cells were fixed, and stained by TRAP. TRAP-positive multinucleated cells containing three or more nuclei were counted (left). Data are expressed as mean ± standard deviation (SD). *$P$<0.05 (the difference from the results with RANKL without LLZ). Microscopic images of TRAP staining in representative cultures are shown (right). Original magnification, x100. (E) RAW264.7 cells were transfected with scrambled (siCTL) or *TAK1* (siTAK1) small interfering RNA, and then cultured with or without RANKL (25 ng/mL) for 24 hours. Cell lysates were then collected, and TAK1, NFATc1 and c-fos protein levels were assayed by western blotting. β-actin served as a loading control. (F) RAW264.7 cells transfected with scrambled (siCTL) or *TAK1* (siTAK1) were cultured in quadruplicate with or without RANKL (25 ng/mL) for 4 days. TRAP-positive multinucleated cells containing three or more nuclei were counted. Data were expressed as mean ± standard devaition (SD). *$P$<0.05. (G) Primary bone marrow cells derived from mice were cocultured in quadruplicate with MM cells as indicated for 4 days with or without LLZ. The cells were then fixed, and stained by TRAP. TRAP-positive multinucleated cells containing three or more nuclei were counted. Data were expressed as mean ± SD. *$P$<0.05.

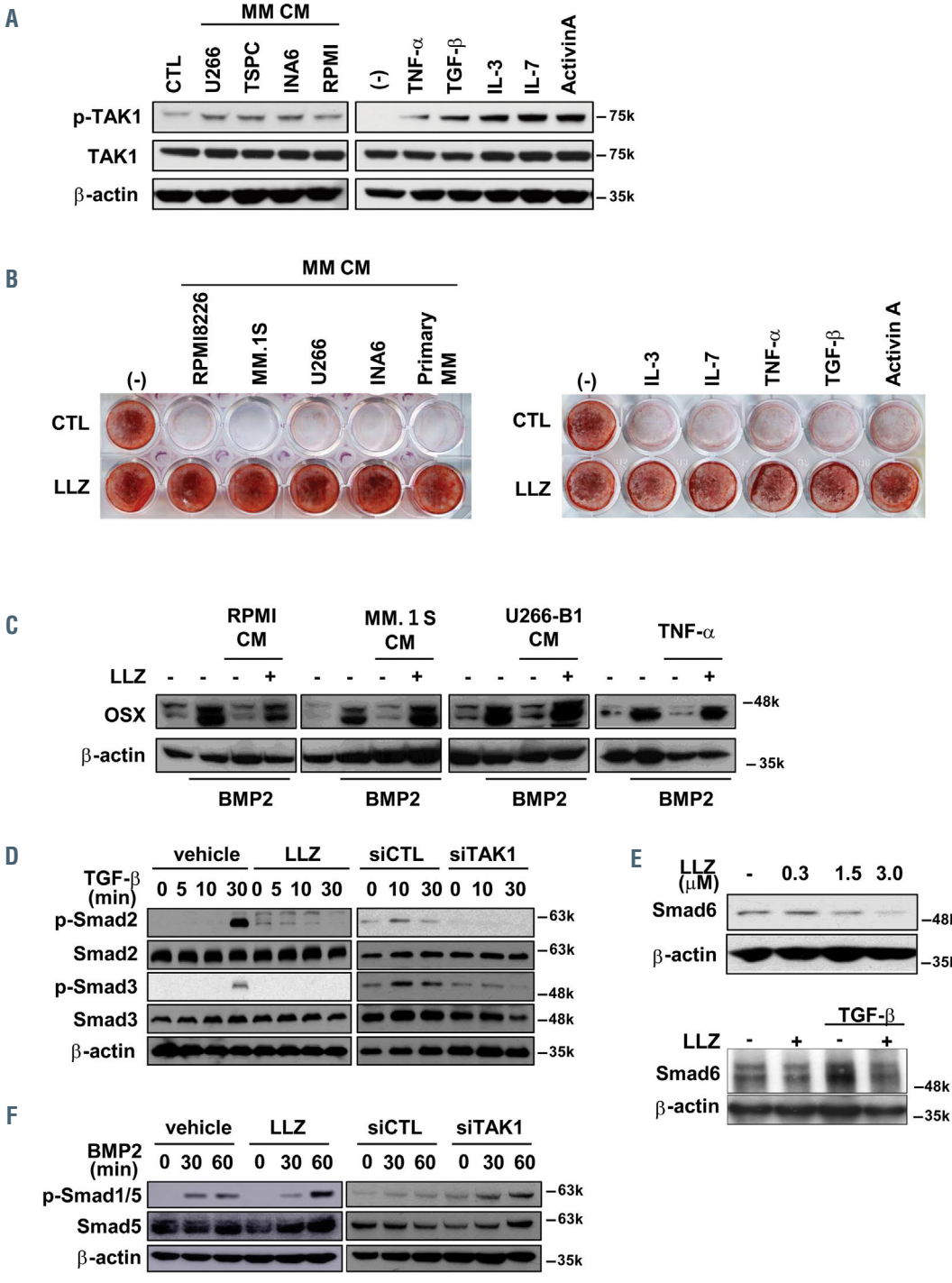


**Figure 5. TAK1 inhibition restores osteoblastogenesis suppressed by multiple myeloma cells as well as major inhibitors for osteoblastogenesis in multiple myeloma.** (A) Conditioned media from the indicated multiple myeloma (MM) cell lines (MM CM) at 25%, or cytokines including TNF-α (1 ng/ml), TGF-β (10 ng/ml), IL-3 (10 ng/ml), IL-7 (10 ng/mL), or activinA (10 ng/mL) were added onto cultures with the MC3T3-E1 cells. After culturing for 24 hours, cell lysates were collected, and phosphorylated TAK1 (p-TAK1) and TAK1 levels were analyzed using western blotting. β-actin served as a loading control. (B) MC3T3-E1 cells were cultured in the presence or absence of LLZ (0.3 μM) in osteogenic media with BMP-2 (25 ng/mL) in 24-well culture plates. MM cells CM from the indicated cell lines and primary MM patient were added at 25%. TNF-α (1 ng/mL), TGF-β (10 ng/mL), IL-3 (10 ng/mL), IL-7 (10 ng/mL), or activinA (10 ng/mL) were added to the indicated wells. After culturing for 14 days, the cells were fixed and mineralized nodule formation was visualized using Alizarin red staining. (C) MC3T3-E1 cells were cultured for 4 days with MM CM (25%) or TNF-α (1 ng/mL) in the presence or absence of LLZ (0.3 μM) in osteogenic media with BMP-2 (25 ng/mL). Then, cell lysates were collected and Osterix (OSX) expression was assayed using western blotting. (D) MC3T3-E1 cells were starved in α-MEM with 1% FBS for 12 hours, and then treated with or without LLZ at 0.3 μM for 3 hours, or transfected with scrambled (siCTL) or *TAK1* (siTAK1) small interfering RNA. TGF-β was added at 10 ng/mL, and cell lysates were harvested after the indicated time periods. The expression of indicated proteins were analyzed using western blotting. (E) (Upper) MC3T3-E1 cells were treated with LLZ at the indicated concentrations for 12 hours. (Lower) MC3T3-E1 cells were treated with LLZ for 30 minutes prior to adding TGF-β (10 ng/mL), then cultured for 12 hours. The expression of Smad6 protein was analyzed using western blotting. (F) MC3T3-E1 cells were starved with or without LLZ at 0.3 μM for 12 hours, or transfected with scrambled (siCTL) or *TAK1* (siTAK1) siRNA. BMP-2 was added at 25 ng/mL, and cell lysates were harvested after the indicated time periods. The expression of indicated proteins were analyzed using western blotting.

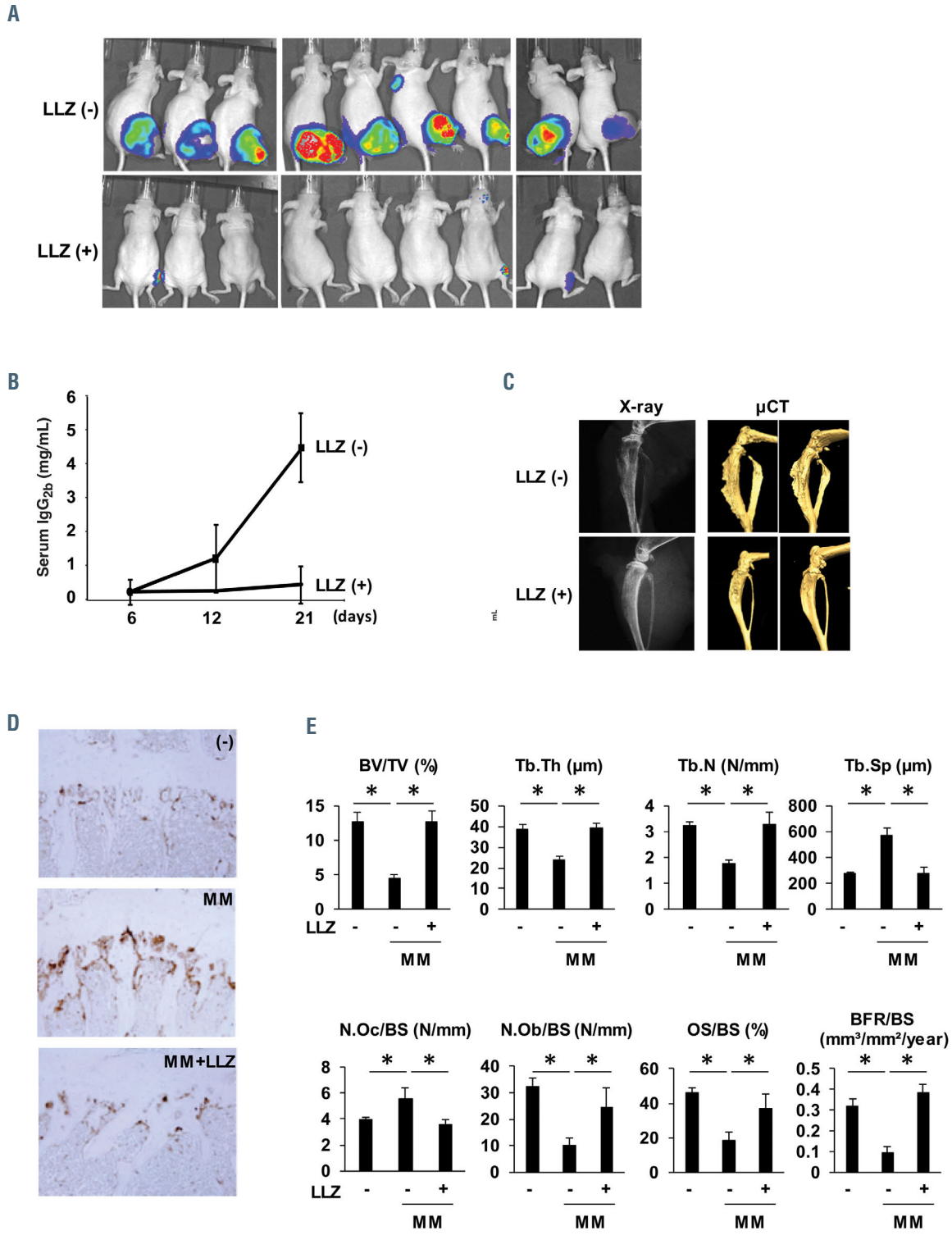


**Figure 6. TAK1 inhibition suppresses multiple myeloma tumor growth and bone destruction *in vivo*.** The mice with intra-tibial inoculation of *luciferase*-transfected 5TGM1 were treated with LLZ at 20 mg/kg or saline intraperitoneally every other day from days 6 to 21. (A) Tumor sizes were assessed at day 21 via *in vivo* bioluminescence measurement using the IVIS Imaging System. For the luciferase detection imaging, 100 μL of 15 mg/mL D-luciferin in phosphate-buffered saline was injected intraperitoneally before taking images. (B) Serum levels of mouse $IgG_{2b}$ were measured over time. (C) The tibiae with the tumor or control ones were extracted, and analyzed using soft X-ray (left) and μ-computed tomography (right) imaging. (D) Immunohistochemical detection of cathepsin K in the tibia from a mouse inoculated with 5TGM1 multiple myeloma (MM) cells. (E) The tibiae were taken out at day 21 and analyzed histomorphometrically. The ratios of the bone volume to total volume (BV/TV), trabecular thickness (Tb.Th), trabecular numbers (Tb.N), trabecular separation (Tb.Sp), number of osteoclasts to bone surface (N.Oc/BS), number of osteoblasts to bone surface (N.Ob/BS) osteoid surface to bone surface (OS/BS), and bone formation rate to bone surface (BFR/BS) were analyzed using sections from three mice in each study arm. Data are expressed as mean ± standard deviation. **P*<0.05.

on bone metabolism in MM, we histomorphometrically analyzed bone lesions in the mouse models. In vehicle-treated mice, bone volume over total volume (BV/TV), trabecular thickness (Tb.Th), trabecular numbers (Tb.N), number of osteoblast surface over bone surface (N.Ob/BS), osteoid surface over bone surface (OS/BS), and bone formation rate over bone surface (BFR/BS) were decreased, whereas trabecular separation (Tb.Sp) and the number of OC surface over bone surface (N.Oc/BS) were increased (Figure 6E). However, treatment with LLZ improved these changes in the 5TGM1-inoculated tibiae. These results demonstrate that the TAK1 inhibition not only suppresses osteoclastic bone destruction but also restores osteoid and bone formation in MM bone lesions. In order to further clarify the direct roles of TAK1 inhibition on pathological bone loss without tumor cells, we investigated the effects of LLZ on bone loss in ovariectomized (OVX) mice. In vehicle-treated OVX mice, bone loss was revealed in μ–CT; and BV/TV, Tb.Th and Tb.N were decreased, whereas Tb.Sp was increased in bone morphometric analysis (*Online Supplementary Figure S7*). However, treatment with LLZ was able to prevent OVX-induced pathological changes, suggesting a protective action of TAK1 inhibition on non-malignant bone loss.

## Discussion

Although MM cells perturb bone metabolism with bone destruction, crosstalk between MM cells and the microenvironment in bone lesions leads to a progressive vicious cycle of tumor growth and bone destruction. The present study demonstrated that TAK1 plays a critical role in the vicious cycle, and suggested that TAK1 is a pivotal therapeutic target to disrupt the key signal transduction pathways responsible for tumor progression and bone destruction in MM. TAK1 activation appears to govern the expression of PIM2 in MM cells and osteoclastic as well as osteoblastic lineage cells; the TAK1-PIM2 signaling pathway is critical for MM tumor expansion and bone destruction. In addition to PIM2 upregulation, TAK1 activation induced Sp1 expression in MM cells. Sp1 is a ubiquitous zinc-finger transcription factor that binds guanine–cytosine-rich elements in the promoter region of its target genes and upregulates various important genes for cancer initiation and progression.[46,47] Sp1 is known to be constitutively overexpressed in many cancers and is associated with poor prognosis.[46] Sp1 expression and its DNA binding activity has been also demonstrated to be upregulated in MM cells.[25] We and others reported that inhibition of Sp1 expression with Sp1 siRNA markedly induced apoptosis in MM cells, indicating that Sp1 as a novel therapeutic target for MM.[25-27] Our results showed that TAK1 activation contributes to Sp1 over-expression in MM cells, and that TAK1 inhibition reduces Sp1 expression to impair MM cell growth and survival. TAK1 inhibition was found to reduce Sp1-mediated IRF4 expression in MM cells. As IMiD have been reported to downregulate IRF4 expression though degradation of IKZF1/3,[23,24] TAK1 inhibition may synergize the downregulation of IRF4 expression in combination with IMiD. TAK1 was also demonstrated to play a critical role in facilitating MM cell-BMSC adhesion via VLA-4-VCAM-1 interaction. TAK1 inhibition reduced VCAM-1 expression in BMSC upregulated by MM cells or TNF-α, and impaired MM cell adhesion to BMSC. MM cell-BMSC adhesion induced IL-6 production and RANKL expression in BMSC in a manner dependent on TAK1 activation. Given that the adhesion of MM cells to BMSC via VLA-4-VCAM-1 interaction confers CAM-DR in MM cells[29,48,49] and osteoclastogenesis,[50] these results suggested the therapeutic impact of TAK1 inhibition on CAM-DR as well as osteoclastogenesis induced by the MM-bone marrow interaction.

RANKL plays an important role in osteoclastogenesis enhanced in MM. As reported previously,[34] RANKL induced the phosphorylation of TAK1 in RAW264.7 preosteoclastic cells in parallel with the degradation of IκBα and phosphorylation of p38MAPK and ERK (Figure 4A). However, TAK1 inhibition abolished these changes in the RANKL-mediated intracellular signaling and suppressed the formation of TRAP-positive OC from bone marrow cells upon treatment with exogenous RANKL (Figures 4D, F) as well as in cocultures with MM cells (Figure 4G). Together with suppression of the TAK1-dependent induction of RANKL expression in BMSC (Figure 3G), TAK1 inhibition can reduce osteoclastogenesis enhanced in MM through blockade of RANKL-mediated signaling in osteoclastic lineage cells. As for osteoblastogenesis, major inhibitors for osteoblastogenesis in MM, including IL-3, IL-7, TNF-α, TGF-β, and activinA, as well as MM cell CM-induced TAK1 phosphorylation while suppressing osteoblastogenesis in MC3T3-E1 preosteoblastic cells; however, TAK1 inhibition restored their osteoblastogenesis (Figures 5A,B). Taken together, these results underscored the value of TAK1 inhibition for preventing progression of bone destruction and restoring bone formation in MM. Finally, we validated the therapeutic effects of TAK1 inhibition *in vivo*. Treatment with LLZ markedly suppressed MM tumor growth and prevented bone destruction in mouse MM models with intra-tibial injection of 5TGM1 MM cells (Figure 6). Although various bone-modifying agents have been developed, bone loss still remains a serious unmet issue in patients with MM; bone formation is hard to be restored in MM bone lesions by clinically available anti-resorptive agents, namely zoledronic acid and denosumab. In contrast to these agents, TAK1 inhibitors appear to be bone anabolic and anti-resorptive agents with tumor-suppressing activity, which may bring considerable benefits for patients with malignant diseases exhibiting bone loss, such as MM patients. Moreover, because TAK1 inhibition is a novel mechanism of action, combination with TAK1 inhibition can improve the therapeutic efficacy of currently available anti-cancer agents while preventing cancer-related and cancer treatment-induced bone loss. Therefore, TAK1 inhibition may be a promising therapeutic option with anti-tumor and bone modifying action, targeting the interaction between MM cells and their surrounding microenvironment. The present results warrant further study for development of novel TAK1 inhibitors useful for MM treatment. We are currently synthesizing novel compounds with better specificity for TAK1 with less toxicity. Further translational research will elucidate the dividends they may yield in improved clinical outcomes.

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***Contributions***

*JT and MA designed the research and conceived the project; PCR was performed by JT, HT, AO and SS; flow cytometry by AO, MH and TH; immunoblotting by JT, HT, MH, AO, AB, TH, SN, MA, SS and MI; transfection by JT, HT, MH, AO and TH; and cell cultures by JT, HT, MH, AO, AB, TH, SN, MA, SS, MI, KS, MO, SF, KK and HM, JT, HT, MH, AO, TH, SN, MH, IE, TH, TM, and MA analyzed the data; JT and MA wrote the manuscript.*

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