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AMP-activated protein kinase mediates lipopolysaccharide-induced proinflammatory responses and elevated bone resorption in differentiated osteoclasts

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Abstract

Systemic and intracellular metabolic states are critical factors affecting immune cell functions. The metabolic regulator AMP-activated protein kinase (AMPK) senses AMP levels and mediates cellular responses to energy-restrained conditions. The ubiquitously expressed AMPK participates in various biological functions in numerous cell types, including innate immune cell macrophages and osteoclasts, which are their specialized derivatives in bone tissues. Previous studies have demonstrated that the activation of AMPK promotes macrophage polarization toward anti-inflammatory M2 status. Additionally, AMPK acts as a negative regulator of osteoclastogenesis, and upregulation of AMPK disrupts the differentiation of osteoclasts. However, the regulation and roles of AMPK in differentiated osteoclasts have not been characterized. Here, we report that inflammatory stimuli-regulated-AMPK activation of differentiated and undifferentiated osteoclasts in opposite ways. Lipopolysaccharide (LPS) inhibited the phosphorylation of AMPK in macrophages and undifferentiated osteoclasts, but it activated AMPK in differentiated osteoclasts. Inactivating AMPK decreased cellular responses against the activation of toll-like receptor signaling, including the transcriptional activation of proinflammatory cytokines and the bone resorption genes *TRAP*, and *MMP9*. The elevation of bone resorption by LPS stimulation was disrupted by AMPK inhibitor, indicating the pivotal roles of AMPK in inflammation-induced activities in differentiated osteoclasts. The AMPK activator metformin did not increase proinflammatory responses, possibly because other factors are also required for this regulation. Notably, changing the activation status of AMPK did not alter the expression levels of bone resorption genes in unstimulated osteoclasts, indicating the essential roles of AMPK in cellular responses to inflammatory stimuli but not in the maintenance of basal levels. Unlike its M2-polarizing roles in macrophages, AMPK

was not responsive to the M2 stimulus of interleukin-4. Our observations revealed differences in the cellular properties of macrophages and osteoclasts as well as the complexity of regulatory mechanisms for osteoclast functions.

1 | INTRODUCTION

Tissue-resident macrophages are key players in immune surveillance and inflammation induction; they are present in the majority of tissues in the body.[1,2] These cells display phenotypical heterogeneity and are thus further classified into distinct subsets based on their micro-anatomical niche. Most of these subtypes share common features, such as phagocytotic capacities and cytokine secretion for regulating innate and adaptive immune responses and mediating wound repair.[3,4] Three types of macrophages populate bone and bone marrow: bone marrow–derived macrophages, osteal macrophages, and osteoclasts.[5,6] Osteoclasts are generally recognized as terminally differentiated "bone macrophages" with multiple myeloid origins, including monocytes, macrophages, dendritic cells, and monocytic progenitors.[7] Cells in the bone microenvironment, such as stromal cells and osteoblasts, trigger the differentiation of osteoclast progenitors in bone tissues by releasing receptor activator of nuclear factor kappa-B ligand (RANKL) and macrophage colony–stimulating factor (M-CSF). The activation of RANKL-RANK signaling induces cell fusion, leading to the formation of multinucleated osteoclasts.[8] As monocytic lineage–derived cells, osteoclasts participate in the innate immune response and regulate the functions of other immune cells.[7] Additionally, the differentiation and bone resorption activities of osteoclasts are regulated by immunological signals. For example, both the activation status and bone resorption activities of osteoclasts are elevated by proinflammatory M1 stimuli.[9] By contrast, anti-inflammatory M2 stimuli inhibit osteoclastogenesis and osteoclast activity.[10]

Osteoclast-mediated bone resorption is regulated by various systemic factors, including inflammation, cytokine and hormone levels, and metabolic status.[11,12] As a critical regulator of metabolic programming and inflammatory signaling, AMP-activated protein kinase (AMPK) might play prominent roles in modulating osteoclast functions. AMPK is a critical enzyme regulating energy homeostasis, metabolic stress, and mitochondrial dynamics.[13] Cellular AMP/ATP ratio determines the activation status of AMPK to further regulate the switch between ATP-consuming anabolic

pathways and ATP-producing catabolic pathways.[14] As a ubiquitously expressed protein, AMPK acts as an essential regulator in innate immune responses and inflammation.[15,16] In macrophages, metabolic reprogramming alters cellular functions such as cytokine production, phagocytosis, and antigen presentation.[17] 5-Aminoimidazole-4-carboxamide ribose (AICAR), an AMPK activator, was reported to reduce the severity of experimental autoimmune encephalomyelitis in an autoimmune mouse model.[18] Stimulating macrophages with anti-inflammatory cytokines resulted in the rapid phosphorylation of AMPK, whereas treating macrophages with a proinflammatory stimulus, namely lipopolysaccharide (LPS), led to AMPK dephosphorylation.[15] The inhibition of AMPKα1 through RNA interference or through treatment with a dominant negative variant dramatically elevated LPS-induced inflammatory responses in macrophages. By contrast, constitutively expressing AMPKα1 resulted in decreased LPS-induced cytokine production and elevated production of interleukin (IL)-10. Moreover, ablating AMPK diminished IL-10-induced macrophage polarization and activation of the phosphoinositide 3-kinase (PI3K)/protein kinase B/mammalian target of rapamycin complex 1 and signal transducer and activation of transcription 3-mediated anti-inflammatory pathways in the mouse model,[16] whereas nuclear factor-κB (NF-κB) pathways were inhibited by the activation of AMPK in the macrophage cell line RAW247.6.[19] These data indicate that AMPK acts as a central negative regulator of the inflammatory function of macrophages. The activation of AMPK promotes macrophage polarization toward anti-inflammatory M2 status.

The functions of AMPK in osteoclastogenesis have also been investigated. Downregulating AMPK through RNA interference or treatment with the AMPK inhibitor compound C promoted the generation of multinucleated osteoclasts,[20] whereas overexpressing AMPK suppressed the differentiation of osteoclasts.[21] These results indicate that AMPK is a negative regulator in osteoclastogenesis. Genetic ablation of *Prkaa1* or *Prkaa2*, which encode AMPK α1 and α2 isoforms, respectively, resulted in the reduction of bone mass and an increase in osteoclast number in either mutant. Static and dynamic bone histomorphometric analyses revealed that *Prkaa1*$^{-/-}$ mice

exhibited elevated bone remodeling, as both bone formation and resorption were increased, whereas *Prkaa2*$^{-/-}$ mice exhibited mainly increased bone resorption.[22] However, because AMPK also acts as a positive regulator of bone formation in osteoblasts, the functions of AMPK in bone resorption and the physiology of osteoclasts remain unclear. Here, we investigated the regulation and role of AMPK in differentiated osteoclasts. Our observations identified similarities and differences in the cellular reactions of AMPK in two different yet closely related types of cells. The results also highlight the physiological significance of AMPK and the importance of immunological and metabolic regulatory mechanisms in bone physiology.

2 | MATERIALS AND METHODS

2.1 | The preparation of RANKL-induced osteoclasts from RAW264.7

The murine macrophage cell line RAW264.7 subclone 2 was obtained from American type culture collection (ATCC) (ATCC TIB-71). Cells were grown in Dulbecco's modified eagle medium (DMEM) (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% (v/v) phosphate buffer saline (FBS) and 1% (v/v) penicillin–streptomycin solution and incubated at 37°C in 5% CO_2 humidified air. The medium was changed every 3 days. To obtain osteoclasts, RAW264.7 cells were grown for 6 days in DMEM containing 10% FBS, 1% P/S, and 50 ng/ml mRANKL (PeproTech).[23] LPS (Sigma-Aldrich L3024) is used as a proinflammatory stimulant at a concentration of 1 μg/ml. Dorsomorphin (compound C, Sigma-Aldrich P5499) and metformin (Sigma-Aldrich) are AMPK inhibitor (10 μM) and activator (2 mM), respectively.

2.2 | The preparation of murine primary osteoclasts

The bone marrow cells were isolated from femurs and tibiae of C57BL/6 mice of 4–6 weeks of age. All mice were housed in a controlled temperature and 12/12 h light dark cycle room. Water and food were provided ad libitum. After collecting of bone and bone marrow, the cells were treated with red blood cell lysis buffer (155 mM NH_4Cl, 12 mM $NaHCO_3$, and 0.1 mM EDTA) and stop reaction with PBS. Next, bone marrow cells were pass through a cell strainer (70 μm) to collect the cells. The primary osteoclast culture was followed the methods of Chevalier et al.[24] with some modifications.

Differentiation occurred over 6–7 days with every other day media change. The differentiated osteoclasts were cultured in basal medium containing M-CFS and RANKL. All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of National Chengchi University. All experiments were performed in accordance with the Guideline for The Care And Use of Laboratory Animals, which is issued by Taiwan Council of Agriculture. This study was carried out in compliance with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.

2.3 | Tartrate-resistant acid phosphatase (TRAP) staining

RAW 264.7 cells were suspended in DMEM containing 10% FBS, 1% (v/v) penicillin-streptomycin solution and plated at 5×10^4 cells/well in a 24-well tissue culture plate with 50 ng/ml RANKL at 37°C in 5% CO_2 humidified air. The medium was replaced every 2–3 days. After 6 days, cells were fixed and stained using the TRAP activity staining kit according to the manufacturer's instructions. TRAP-positive cells appeared dark red and TRAP-positive multinucleated cells with more than three nuclei were counted.[25,26]

2.4 | Reverse transcription quantitative PCR (RT-qPCR) assay

Total RNA was extracted using the RNAzol reagent (Mrcgene; Molecular Research Center, Inc.) according to the manufacturer's protocol. Complementary DNA (cDNA) was synthesized using an IQ2 MMLV RT-Script kit (Bio-Genesis Technologies Inc.) according to the manufacturer's protocol. qPCR was performed using SYBR-Green (Applied Biosystems, Thermo Fisher Scientific, Inc.), and data collection was conducted using an ABI 7300 (Applied Biosystems; Thermo Fisher Scientific, Inc.). The PCR cycling conditions were as follows: 95°C for 2 min, followed by 40 cycles at 95°C for 20 s, 58°C or 53°C for 20 s, and 72°C for 30 s, with a final extension step of 95°C for 15 s, 60°C for 1 min, 95°C for 15 s, and 60°C for 15 s. Primer sequences were as follows: GAPDH:

Forward: GCACAGTCAAGGCCGAGAAT
Reverse: GCCTTCTCCATGGTGGTGAA
tumor necrosis factor-α (TNF-α):
Forward: CCCTCACACTCAGATCATCTTCA
Reverse: GCTACGACGTGGGCTACAG
TRAP:
Forward: GCTGGAAACCATGATCACCT
Reverse: GAGTTGCCACACAGCATCAC
ctsk:

Forward: CTTCCAATACGTGCAGCAGA
Reverse: TCTTCAGGGCTTTCTCGTTC
MMP9:
Forward: GTTTTTGATGCTATTGCTGAGATCCA
Reverse: CCCACATTTGACGTCCAGAGAAGAA
Arg1:
Forward: GAATCTGCATGGGCAACC
Reverse: GAATCCTGGTACATCTGGGAAC
iNOS:
Forward: CTTTGCCACGGACGAGAC
Reverse: TCATTGTACTCTGAGGGCTGA
PRKAA1:
Forward: GTCAAAGCCGACCCAATGATA
Reverse: CGTACACGCAAATAATAGGGGTT

GAPDH was used as an internal control for normalization. Gene expression was calculated using the delta Ct method.

2.5 | Western blot analysis

Protein expression was analyzed through Western blot analysis. The cell lysate was extracted using a lysis buffer. Samples with a final protein amount of 20 μg were loaded onto a sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel (NuSep, Bio-Rad) and were electrophoresed. Subsequently, the separated proteins were transferred onto polyvinylidene difluoride membranes, which were immunoblotted using p-AMPK (Cell Signaling #9910 sampler, 1:1000), AMPK (Cell Signaling #9926 sampler, 1:1000), p-p44/42 (Cell Signaling #9910 sampler, 1:2000), p44/42 (Cell Signaling #9926 sampler, 1:1000), phosphorylated stress-activated protein kinase/Jun amino terminal kinase (p-SAPK/JNK) (Cell Signaling #9910 sampler, 1:1000), SAPK/JNK (Cell Signaling #9926 sampler, 1:1000), phosphorylated inhibitor of nuclear factor kappa-B kinase subunit alpha/beta (p-IKKa/b) (Cell Signaling #2697, 1:1000), p-p38 (Cell Signaling #9910 sampler, 1:1000), p38 (Cell Signaling #9926 sampler, 1:1000), p-p65 (Cell Signaling #3303 sampler, 1:1000), p65 (Cell Signaling #8242 sampler, 1:1000), and beta-actin primary antibodies overnight at 4°C and then incubated with a horseradish peroxidase (HRP) or HRP-labeled secondary antibody. The protein bands were visualized using the enhanced chemiluminescence method.

2.6 | Pit formation assays

Bone resorption activity was measured by Bone Resorption Assay Kit (CosMo Bio). In summary, mouse bone marrow cells collected from tibia and femur were plated on a calcium phosphate-coated 48-well plate (2×10^4 cells/well) in the presence of M-CSF (30 ng/ml) and RANKL (100 ng/ml) with or without LPS (1 μg/ml) and compound C (10 μM) for 7 days. Remove the cells by treating the plate with 5% sodium hypochlorite for 3–5 min. The pit area was photographed under microscope and calculated with ImageJ software.
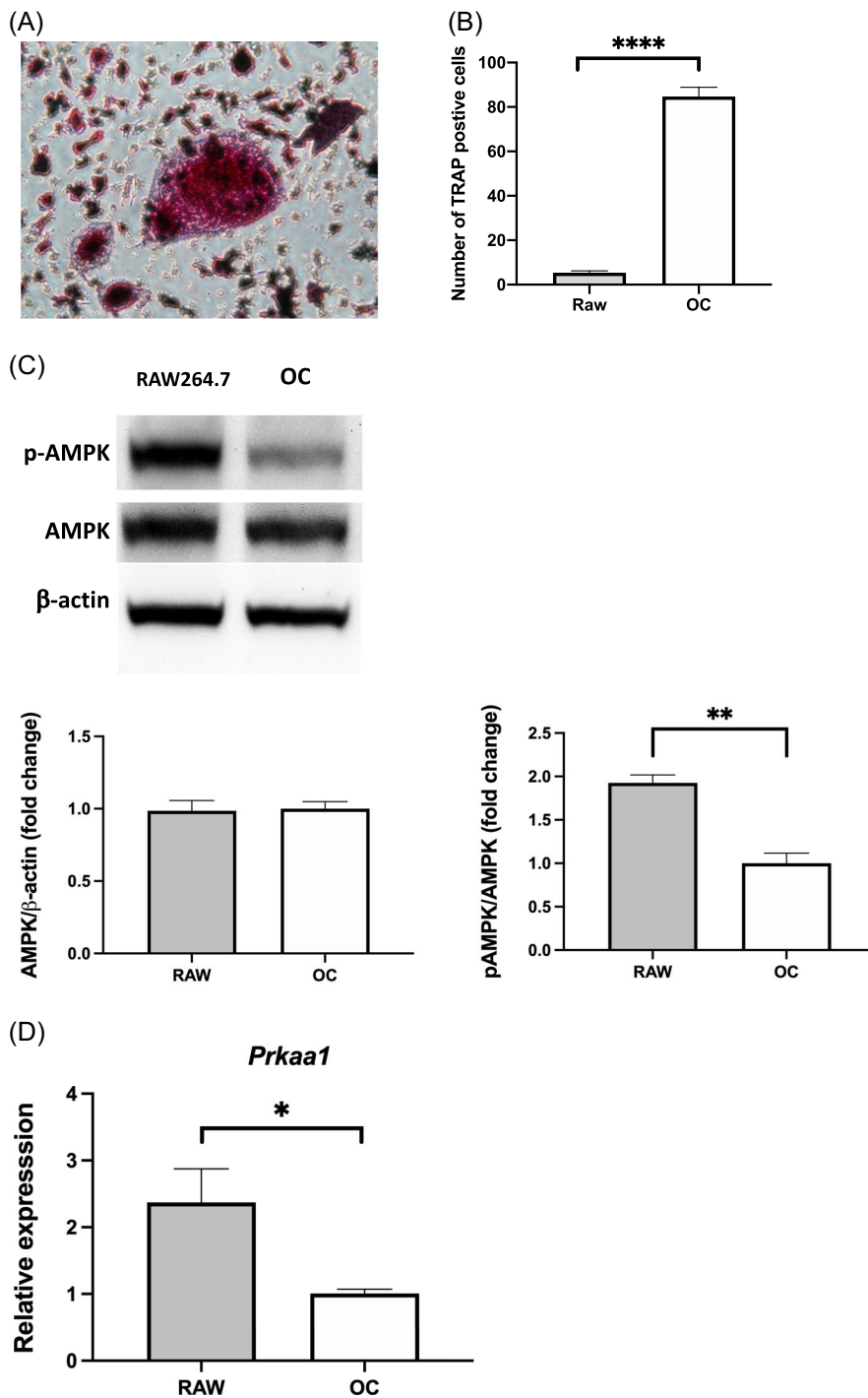
2.7 | Statistical analysis

Data are expressed as pooled means ± standard errors of the mean (SEMs) of at least three independent experiments. Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test or Student's t test (GraphPad Prism software). p values of <.05, <.002, <.001, and <.0001 were considered statistically significant and are indicated as *, **, ***, and ****, respectively.

3 | RESULTS

3.1 | Phosphorylation of AMPK was inhibited during osteoclastogenesis

To study the functional roles of AMPK in differentiated osteoclast and bone resorption, two cell models were employed to examine the AMPK activation and the expression of bone resorption genes, as well as the activation of bone resorption-related signaling pathways. Primary osteoclasts were produced by treating mouse bone marrow cells with M-CSF and RANKL. In parallel, RAW264.7 macrophages were used as precursor osteoclasts, and the differentiation into osteoclasts can be induced with RANKL. The degree of differentiation was verified by staining of TRAP (TRAP staining). Figure 1A,B demonstrated the giant TRAP-positive multi-nucleated cells generated from fusion of precursor cells, showing that differentiated osteoclasts can be induced by our protocol. Previous studies have revealed that the activation of AMPK suppresses osteoclastogenesis, implying that the activation of AMPK might be negatively regulated during differentiation. Two isoforms of the catalytic subunits, AMPKα1 and α2, have been identified. Only AMPKα1 is expressed in macrophages and differentiated osteoclasts. Thus, in this study, we focused on analyzing the AMPKα1 protein and its phosphorylation levels. Figure 1C,D demonstrated the phosphorylation levels of AMPKα1 and the expression levels of Prkaa1 gene of the differentiated osteoclast from the RANKL-induced RAW264.7 cells. Compared with untreated cells, the phosphorylation of AMPK was significantly reduced after RANKL stimulation (Figure 1C). The results from the quantitative PCR of Prkaa1 genes

FIGURE 1   The activation of AMPK was downregulated during osteoclastogenesis. The expression of TRAP in primary osteoclasts (A). Panel (B) represents the percentage of TRAP staining positive cells in RANKL-induced differentiated osteoclasts. The phosphorylation of AMPK was inhibited in differentiated osteoclasts (C). The expression of Protein Kinase AMP-Activated Catalytic Subunit Alpha 1 (*Prkaa1*) genes was decreased in differentiated osteoclasts (D). Each column represents the mean $\pm$ SEM of at least three independent experiments. Symbols indicate significance difference between macrophages and differentiated osteoclasts (*$p < .05$, **$p < .002$, ****$p < .0001$). AMPK, AMP-activated protein kinase; TRAP, tartrate-resistant acid phosphotase

3.2 | LPS stimulation activated AMPK in differentiated osteoclasts

Because osteoclasts are derived from macrophages, both types of cells share common features. Both types can be activated by proinflammatory M1 signals such as

demonstrated that the activation of RANKL-RANK signaling suppressed the expression of AMPK genes (Figure 1D).

lipopolysaccharides (LPS), and inflammatory responses, including the production of proinflammatory cytokines and the generation of nitric oxide (NO), are enhanced.[27] In this study, we then determined whether AMPK is activated if osteoclasts are stimulated. The phosphorylation of AMPK was increased in osteoclasts with LPS stimulation (Figure 2A), suggesting that AMPK is involved in the activation of osteoclast and bone resorption. Both the AMPK protein amount and the expression of the *Prkaa1* gene remained unaltered after stimulation, demonstrating that

inflammatory signals upregulate AMPK only at post-translational levels. Additionally, the transcriptional levels of TNF-α and inducible nitric oxide synthase (iNOS) were elevated upon LPS stimulation in both macrophages and osteoclasts (Figure 2B,C). The expression levels of those two inflammatory genes were increased by a greater extent in differentiated osteoclasts than in macrophages, possibly due to the activation of the downstream RANK, including the nuclear factor kappa B (NF-κB) and the mitogen-activated protein kinase (MAPK) pathways. The elevation of *nos2* gene expression was significantly lower,

whereas the transcriptional upregulation of the cytokine gene *tnfa* was higher in differentiated osteoclasts than in macrophages after LPS treatment. These results imply differences in the cellular properties of the two cell types. We further determined the activation status of both pathways at the presence of LPS in differentiated osteoclasts. MAPKs, including extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinase (JNK) and p38, are important kinases involving in the transcriptional regulation of essential genes of bone resorption, such as *ctsk*, *TRAP*, and *MMP9*.[28] Figure 2D–F showed that both
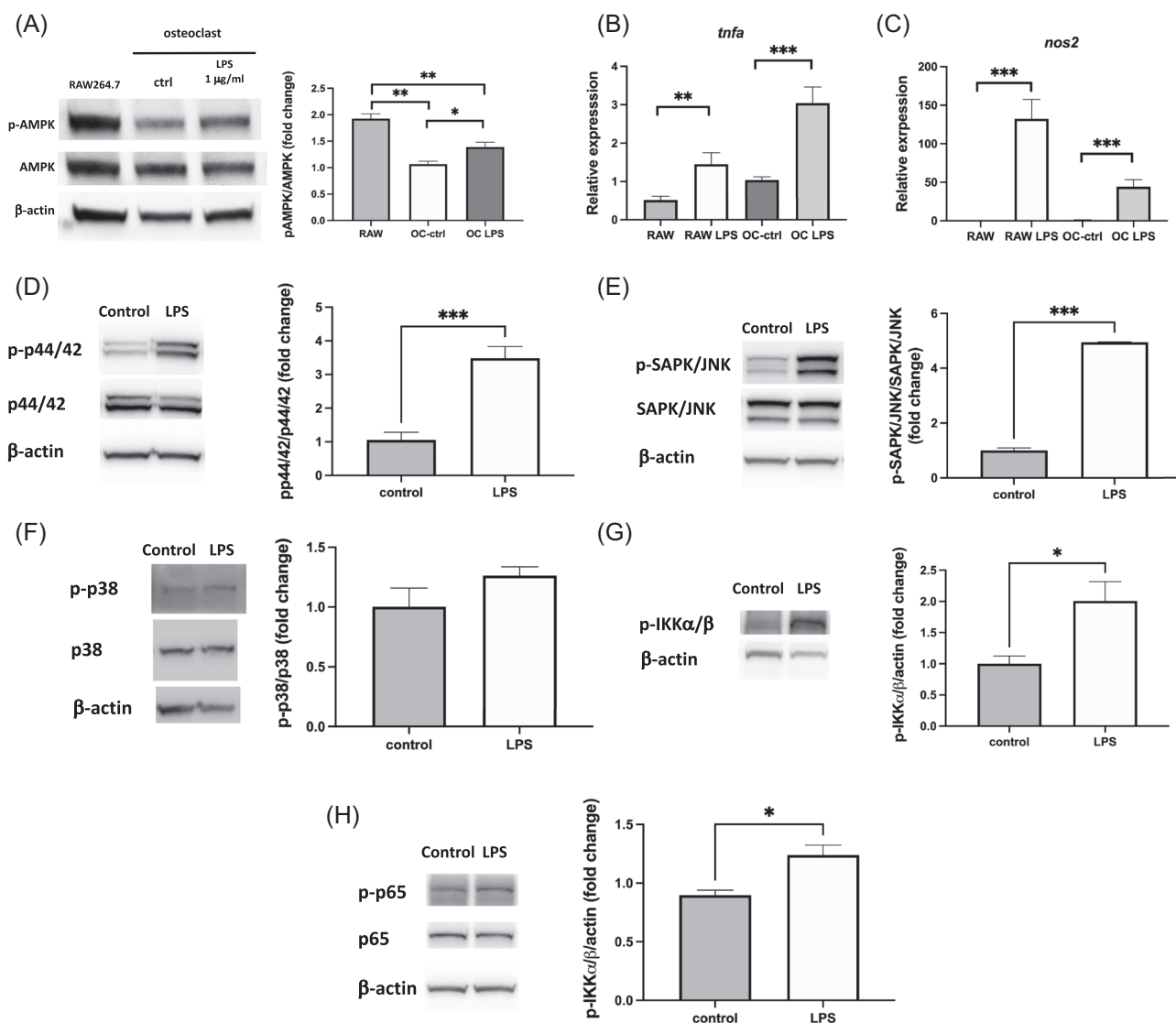

FIGURE 2 The activation of AMPK was increased in the differentiated osteoclast after LPS treatment. The phosphorylation of AMPK was increased with LPS stimulation in osteoclasts (A). Quantitative levels of inflammatory genes TNF-α (B) and iNOS (C) expression were measured by qPCR in osteoclasts after exposure to 1 μg/ml LPS for 6 h. The MAPK (D–F: ERK (D), JNK (E), and p38(F)) and NF-κB (G,H; IKKα/β (G) and p65(H)) pathways were activated with LPS stimulation. Each column represents the mean ± SEM of at least three independent experiments. Symbols indicate significance difference between treatment with/without LPS (*$p < .05$, **$p < .002$, ***$p < .001$). AMPK, AMP-activated protein kinase; ERK, extracellular signal-regulated kinases; iNOS, inducible nitric oxide synthase; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor kappa B; qPCR, quantitative polymerase chain reaction; TNF-α, tumor necrosis factor-α

ERK and JNK are activated by LPS stimulation. Additionally, the enhancement of phosphorylation of p65 and IκB kinase (IKKα/β) indicated that LPS upregulated the inflammatory signaling in the differentiated osteoclasts (Figure 2G,H).

3.3 | AMPK was required for LPS-induced inflammatory responses in osteoclasts

To test whether AMPK is involved in cellular responses to LPS treatment, the expression levels of inflammatory mediator genes were measured with or without the presence of the AMPK inhibitor compound C. Dorsomorphin, also known as compound C (6-[4-(2-Piperidin-1-ylethoxy) phenyl]-3-pyridin-4-ylpyrazolo [1,5-a] pyrimidine) is the most common-used direct AMPK inhibitor with K_i of 109 nM in cell-free assays. This compound exhibits cell permeable, potent, reversible, and selective properties, with no significant inhibition of several structurally related kinases including ZAPK, SYK, PKCθ, PKA, and JAK3.[29] The expression levels of *tnfa* and *nos2* genes of osteoclast differentiated from RANKL-induced RAW264.7 cells and primary osteoclasts are depicted in the Figure 3A–D, respectively. The expression levels of inflammatory genes were generally low, and the treatment of unstimulated osteoclasts with compound C did not alter the expression of either gene. By contrast, the elevation of the expression of two proinflammatory genes was impaired by the inhibition of AMPK activity (Figure 3A,B), demonstrating that activated AMPK is required for inflammatory responses in osteoclasts. In differentiated primary osteoclasts, their responses to LPS and the effects of AMPK inhibitor to stimulated osteoclasts resembled those of RANKL-induced RAW264.7 cells (Figure 3C,D). Our results revealed that the activation of AMPK is a critical regulator for the upregulation of proinflammatory mediators.
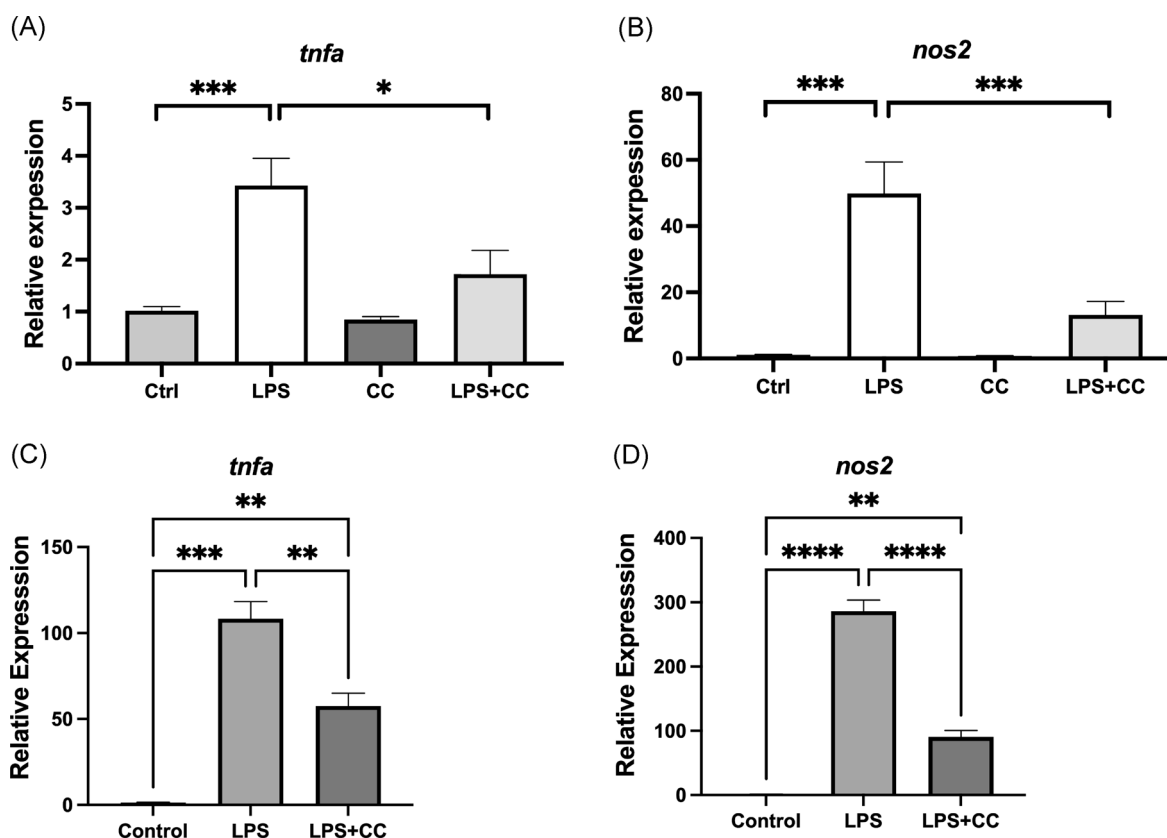

FIGURE 3 The activation of AMPK was required for proinflammatory genes expression in both RANKL-induced osteoclasts and primary osteoclasts after LPS stimulation. Quantitative levels of inflammatory genes TNF-α (A) and iNOS (B) expression were measured by qPCR in RANKL-induced osteoclasts after exposure to 1 μg/ml LPS, compound C (CC) or LPS + CC for 6 h. Quantitative levels of inflammatory genes TNF-α (C) and iNOS (D) expression were measured by qPCR in primary osteoclasts after exposure to 1 μg/ml LPS or LPS + CC. Each column represents the mean ± SEM of at least three independent experiments. Symbols indicate significance difference between treatments (*$p < .05$, **$p < .002$, ***$p < .001$, ****$p < .0001$). AMPK, AMP-activated protein kinase; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; qPCR, quantitative polymerase chain reaction; RANKL, receptor activator of nuclear factor kappa-B ligand; TNF-α, tumor necrosis factor-α

3.4 | Elevation of bone resorption genes by LPS stimulation in osteoclasts was AMPK-dependent

The major cellular functions of osteoclasts are to secrete extracellular matrix–digesting enzymes such as collagenase, cathepsin K (*ctsk*), and matrix metallopeptidase 9 (*MMP9*) as well as bone corrosive acids[28] to mediate bone resorption. The transcriptional regulation of bone resorption genes is one of the main mechanisms regulating bone resorption. This regulation is mediated by the downstream transcription factors in RANKL-RANK pathways, such as nuclear factor of activated T-cells c1 (NFATc1). The role of AMPK in bone resorption was tested by examining the expression of bone resorption genes in differentiated osteoclasts. The expression levels of *ctsk*, *mmp9*, and *trap* were determined when AMPK activation in multinucleated cells was inhibited. LPS stimulation did not elevate the expression of *ctsk* genes, suggesting that the transcriptional regulation of those gene resorption genes is not solely controlled by NFATc1. Inhibition of AMPK did not alter the expression of *mmp9*, and *trap* genes, meaning that AMPK might not participate in regulating bone resorption under normal physiological conditions (Figure 4A–C). Compound C only suppressed the upregulation of the bone resorption genes in differentiated osteoclasts with LPS stimulation. All the data of compound C treatment demonstrated that AMPK serves as a key regulator of cellular responses to inflammatory stimuli in osteoclasts. Moreover, these results suggest that AMPK is involved in the inflammatory upregulation of bone resorption through the transcriptional activation of bone resorption genes. To verify the AMPK functions in bone resorption, the similar set of experiments were also conducted on the differentiated primary osteoclasts. First, the phosphorylation of AMPK was increased upon LPS stimulation. The disruption of the AMPK activation by LPS assured the inhibitory effects of compound C on AMPK activity (Figure 4D). We also examined the expression levels of the bone resorption genes in differentiated primary osteoclasts responding to LPS and the treatment of compound C. Comparable to the response of RANKL-induced RAW264.7 cells, the expression of *TRAP* and *MMP9* genes were enhanced by LPS stimulation, and the upregulation was impaired when AMPK activation was inhibited (Figure 4E–G). To further verify this phenomenon on physiological levels, the pit formation was performed by using primary osteoclasts. Inducing the osteoclasts with proinflammatory stimulus LPS elevated the bone resorption activities, while downregulating AMPK activation inhibited bone resorption (Figure 4H). Combining all the results, we revealed the pivotal roles of AMPK as a positive regulator of inflammation-induced elevation of bone resorption.

3.5 | Enhancing AMPK activation did not affect LPS-stimulated cellular responses in osteoclasts

Metformin is a commonly used AMPK activator. It was applied to increase AMPK activation in this study. Enhancing AMPK activation did not change the expression of either proinflammatory or bone resorption genes under unstimulated conditions (Figure 5A–E). This outcome together with the results of compound C treatment of unstimulated osteoclasts revealed that changing the activation status of AMPK likely does not alter bone resorption. In addition, activating AMPK only showed tendency of strengthening the LPS-induced upregulation in some of the previously mentioned genes, such as *tnfa*, *trap*, and *mmp9*, but not *nos2* or *ctsk* (Figure 5A–E). These results suggest that the inflammatory upregulation of proinflammatory genes and bone resorption genes in osteoclasts might not be mediated by a single protein. This cellular response might be strictly regulated by multiple factors. Intracellular environments also influence cellular phenotypes. Additionally, all the aforementioned results demonstrate that AMPK is a positive regulator of cellular responses to inflammatory signaling in differentiated osteoclasts.

3.6 | AMPK was not responsive to M2 stimulation in differentiated osteoclasts

AMPK can be activated by anti-inflammatory signaling including IL-4 or IL-10 and can further mediate the downstream anti-inflammatory responses in macrophages. We compared AMPK activation and cellular responses between macrophages and osteoclasts. Arginase 1 is commonly considered a marker of anti-inflammatory states. The expression of *arg1* was induced by IL-4 in osteoclasts, confirming that osteoclast responses were inducible by anti-inflammatory signals (Figure 6A). However, the phosphorylation of AMPK was not responsive to IL-4 stimulation (Figure 6B), meaning that the anti-inflammatory responses of osteoclasts are independent of AMPK reactions. Also, the treatment did not affect the expression of the Prkaa1 gene in differentiated osteoclasts (Figure 6C). These results all revealed differences between macrophages and differentiated osteoclasts.

4 | DISCUSSION

Our results revealed a complex network regulating the physiological functions of differentiated osteoclasts. Bone resorption is regulated by biochemical factors in the microenvironments of bone tissues as well as by several systemic regulatory factors, such as hormones,
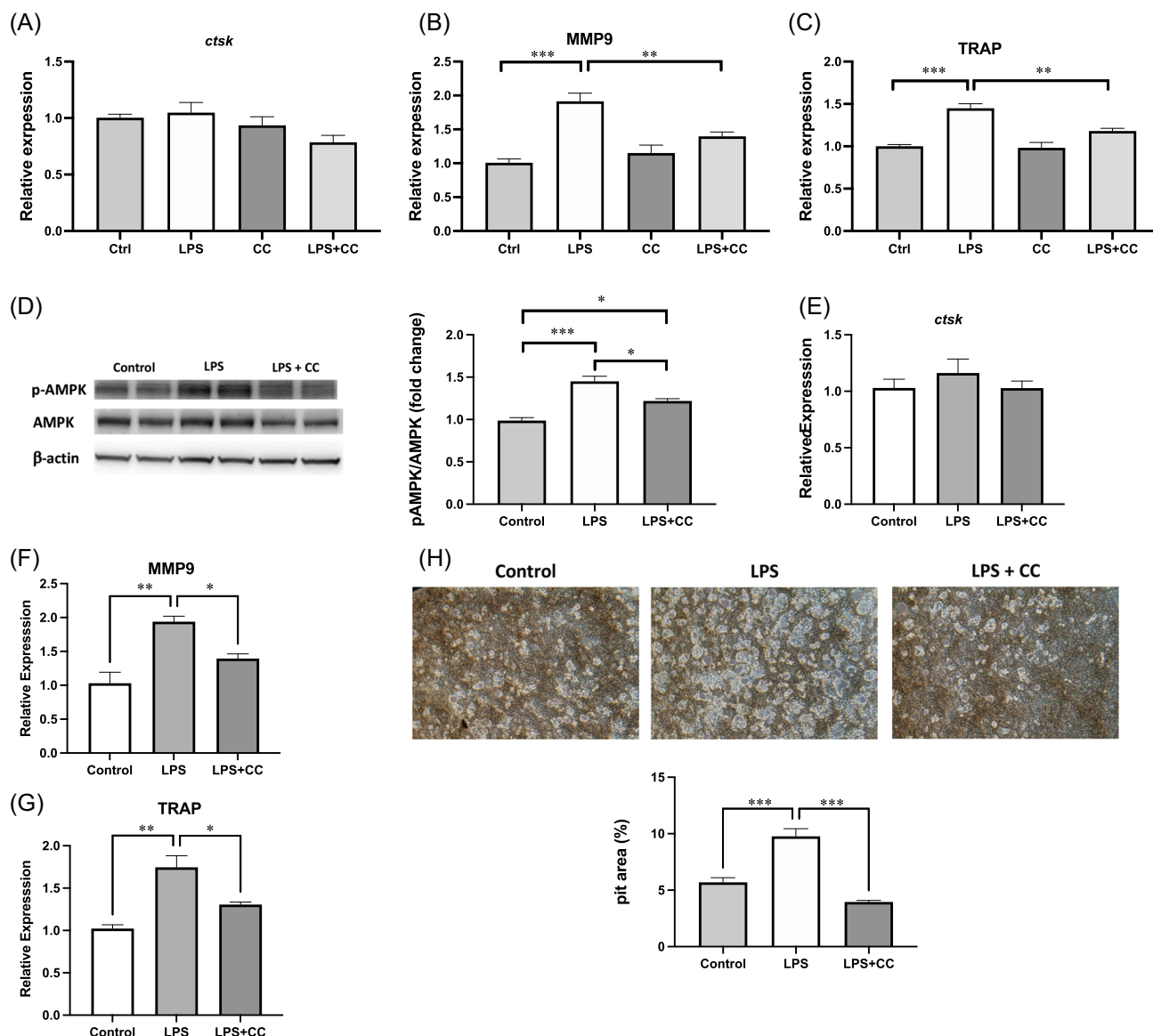
FIGURE 4 The elevations of the expressions of bone resorption genes and bone resorptive activities were AMPK-dependent in LPS-stimulated osteoclasts. The expression of bone resorption genes ctsk (A), MMP9 (B), and TRAP (C), after LPS, compound C (CC) or LPS + CC treatment in osteoclast for 6 h separately. The phosphorylation of AMPK was increased with LPS stimulation in primary osteoclasts (D). Quantitative levels of bone resorption genes ctsk (E), MMP9 (F), and TRAP (G) expression were measured by qPCR in primary osteoclasts after exposure to LPS or LPS + CC. The increase of bone resorptive activity induced by LPS is AMPK dependent (H). Each column represents the mean ± SEM of at least three independent experiments. Symbols indicate significance difference between treatments (*$p < .05$, **$p < .002$, ***$p < .001$). AMPK, AMP-activated protein kinase; LPS, lipopolysaccharide; MMP9, matrix metallopeptidase 9; qPCR, quantitative polymerase chain reaction; TRAP, tartrate-resistant acid phosphatase

immunological signals, and metabolic states of the body.[11,12] AMPK is considered a key metabolic sensor and regulator as well as an essential modulator of macrophage polarization. Its biochemical capacities suggest that this enzyme might be an essential regulator of osteoclast functions. Furthermore, the functions of AMPK can be regulated by inflammatory stimuli and metabolic profile changes at transcriptional and posttranslational levels. The transcriptional controls of AMPK mainly act through

the regulation of the transcription of the catalytic subunits AMPKα1 and α2. In immune cells, only the AMPKα1 gene *Prkaa1* is expressed. The transcription factor binding sites for the Prkaa1 promoter include AP-1, ATF-2, c-Jun, Egr-1, Nkx2-5, STAT1, and STAT1alpha, suggesting that this gene is a target of inflammatory regulation. Our results indicate that the expression levels of the *Prkaa1* gene were inhibited during differentiation but remained unaltered against inflammatory stimuli. In parallel, the
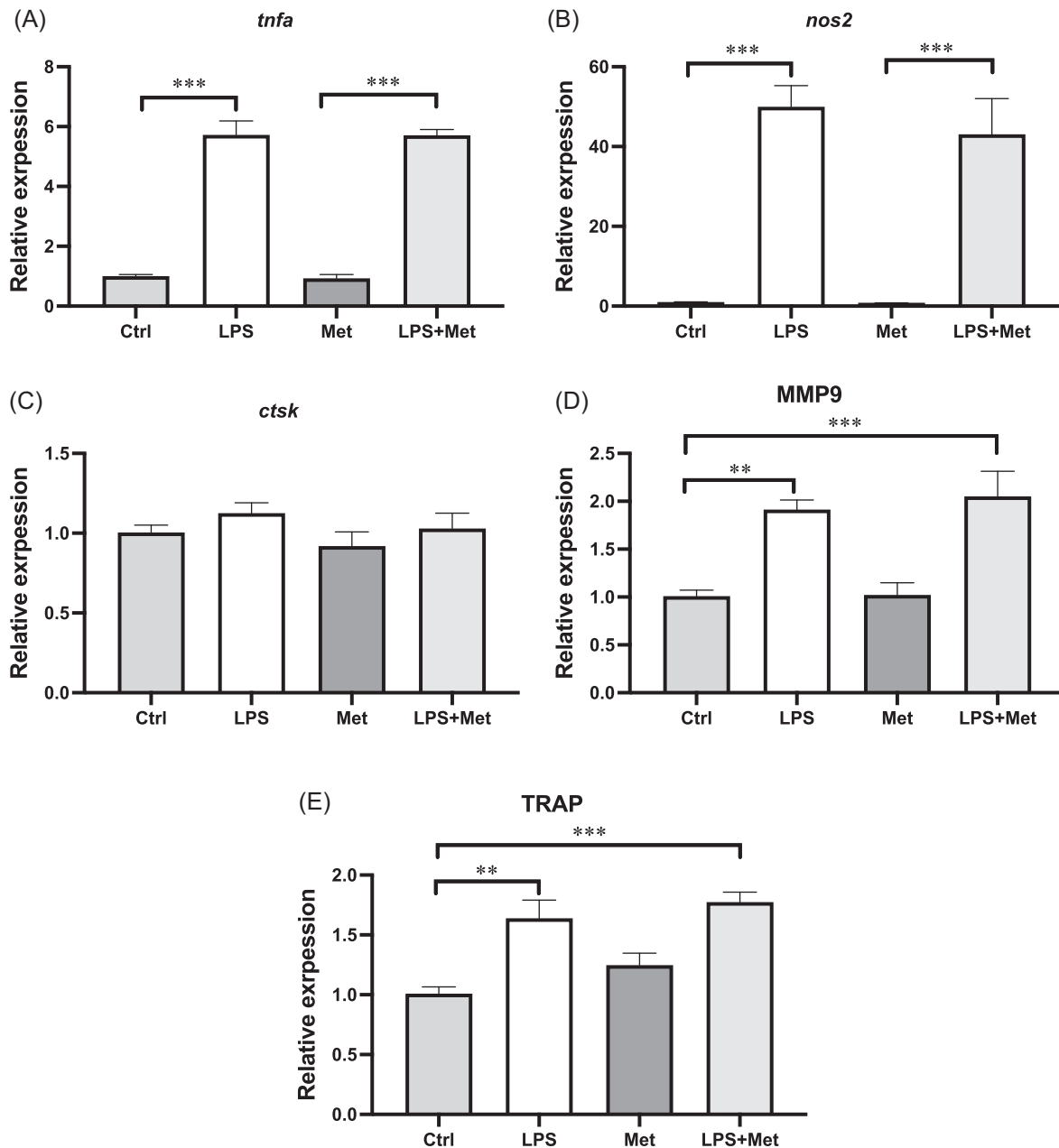
FIGURE 5 Enhancing AMPK activation by metformin increased LPS-stimulated cellular responses in osteoclasts. The expression of proinflammatory genes TNF-α (A) and iNOS (B); and bone resorption genes ctsk (C), MMP9 (D), and TRAP (E) after LPS, compound C (CC) or LPS + CC treatment in osteoclasts for 6 h separately. Each column represents the mean ± SEM of at least three independent experiments. Symbols indicate significance difference between treatments (**$p < .002$, ***$p < .001$). AMPK, AMP-activated protein kinase; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; MMP9, matrix metallopeptidase 9; TNF-α, tumor necrosis factor-α; TRAP, tartrate-resistant acid phosphotase

phosphorylation of AMPK is regulated by various factors. Our data demonstrate that the phosphorylation of AMPK was inhibited during the differentiation of osteoclasts but increased in osteoclasts differentiated by LPS stimulation. These observations imply that AMPK might not be involved in differentiation and bone resorption under normal physiological conditions but might participate in the regulation of osteoclast functions.

Much recent evidence has emphasized the link between osteoimmunology and bone diseases. Immunological factors such as inflammation and cytokine levels have been identified as key regulators of bone resorption and other osteoclast physiological activities.[11] The activation of RANK-RANKL signaling mediates specialized bone resorption activities. The binding of RANKL with RANK recruits tumor necrosis factor
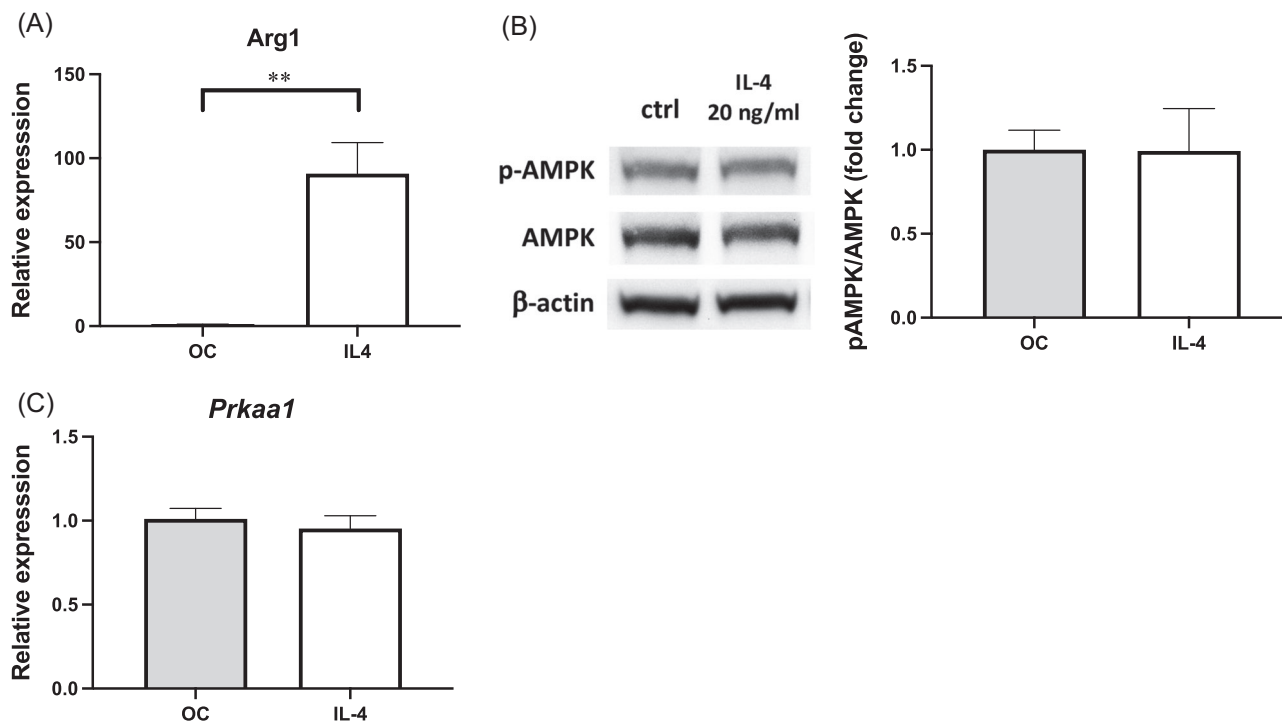
FIGURE 6 The IL-4 induced M2 stimulation was not through the AMPK pathway in the differentiated osteoclasts. Quantitative levels of anti-inflammatory states genes Arg1 (A) expression were measured by qPCR in osteoclast after exposure to IL-4 for 6 h. The phosphorylation of AMPK was not changed in differentiated osteoclasts (B) after M2 stimulation. IL-4 stimulation did not alter the expression of Prkaa1 in differentiated osteoclasts (C). Each column represents the mean ± SEM of at least three independent experiments. ** indicate significance difference between treatment with/without IL-4 where $p < .002$. AMPK, AMP-activated protein kinase; IL-4, interleukin 4; qPCR, quantitative polymerase chain reaction

receptor–associated factors (TRAFs) and further activates several signaling pathways, including the MAPK pathway, phosphatidylinositol-3 kinase (PI3K) pathway, and NF-κB pathway.[30,31] Several transcription factors, such as activator protein-1 (AP-1), NF-κB, and NFATc1, are downstream targets of these activating pathways. The transcriptional profile switch defines the physiological characteristics of osteoclasts. Components of normal and aberrant immune responses might serve as costimulatory signaling through the activation of NFATc1 or other osteoclastogenic transcription factors, enhancing osteoclast functions. Several activators of the NF-κB pathway, such as LPS, have been identified as positive regulators of osteoclast functions, and interleukin-10 (IL-10), IL-4, and other cytokines have been identified as negative regulators.[32,33] Our results agree with the hypothesis that LPS activates MAPK and NF-κB pathways, and consequently enhances proinflammatory responses and bone resorption in differentiated osteoclasts. Additionally, our data demonstrate that AMPK is required for the LPS-induced osteoclast cellular response. Activation of AMPK does not change the expression of proinflammatory or bone resorption genes with or without LPS stimulation, which indicates that other factors are essential for the upregulation of these genes. Interestingly, the bone resorption induced by inflammation appeared to be regulated by different factors. AMPK only involves in the inflammation-induced bone resorption, not under normal physiological conditions. The regulations of AMPK in bone resorption are summarized in Figure 7. Moreover, osteoclasts are bone-residing immune cells, and the immunoregulatory nature of their functions has been demonstrated, including their role in regulating inflammation and T-cell activities. The molecular mechanisms of the immunoregulatory functions remain elusive. Our data suggest that AMPK is a key effector involved in these osteoclast functions.

Our results also revealed substantial differences in molecular landscapes between differentiated osteoclasts and macrophages or undifferentiated osteoclasts, their cellular responses against inflammatory stimuli, and their cellular metabolic states. Osteoclastogenic transcription factors mediate the reprogramming of postfusion osteoclasts by inducing the expression of genes essential for bone resorption, including TRAP and cathepsin K,[34,35] acid producing genes ATPase H1 transporting V0 subunit d isoform 2 (ATP6V0D2), and fusion-specific genes, including dendritic cell-specific
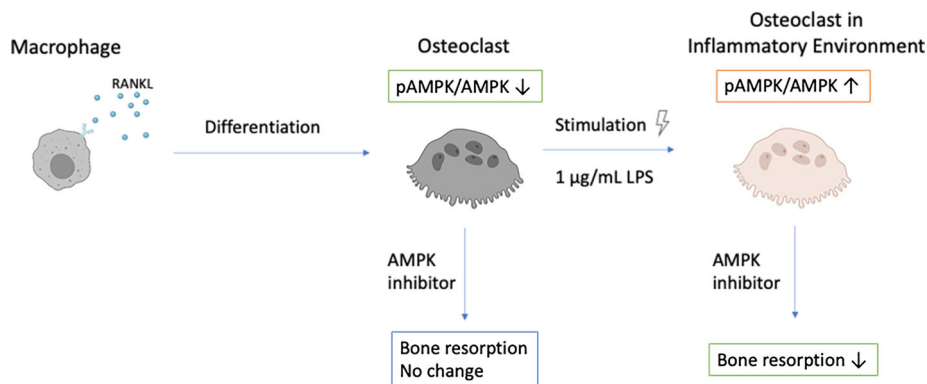
FIGURE 7 AMPK is required for bone resorbing activities of osteoclast in inflammatory conditions. AMPK serves as a negative regulator of osteogenesis and bone resorption in normal conditions. The expression of AMPK is suppressed during RANKL-mediated differentiation. Conversely, inflammatory signals upregulate AMPK expression. AMPK turns out to become an essential player for the bone resorbing activities in inflammatory conditions. AMPK, AMP-activated protein kinase; RANKL, receptor activator of nuclear factor kappa-B ligand

transmembrane protein (DC-STAMP). Also, this differentiation and the cell fusion process are likely to alter gene expression profiles and epigenetic landscapes. Although some basic cellular features remain comparable between pre- and postcell fusion osteoclasts, such as phagocytotic capacities and M1/M2 polarization, the regulatory mechanisms underlying these phenomena might undergo reprogramming. Previous studies have reported that AMPK potentiates M2 polarization in macrophages. Our data revealed different actions of AMPK against pro- and anti-inflammatory stimuli.

Metabolic profiles in osteoclasts are also key determinants of bone resorption. Osteoclast differentiation is associated with increases in number, size, and cristae abundance of mitochondria as well as increased glycolysis and oxidative phosphorylation.[36] Disruption of mitochondrial complex I impaired osteoclast differentiation and led to osteopetrosis in mice.[37] Various metabolism products or metabolites exert direct actions on osteoclast functions. Extracellular proton ions are obligatory precursors of cell fusion and osteoclast activation.[38] Purinergic signalings also modulate osteoclast functions. At physiological concentrations, ATP is a positive regulator of both the formation and activity of osteoclasts.[39] Further characterization of the metabolic regulatory mechanisms, including the cross-talk between metabolic and immunological signaling, involved in osteoclast functions and the involvement of AMPK might be essential to understanding the balance of bone remodeling.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

Yu-Hsu Chen: Investigation, Data curation, Visualization, Writing-original draft, Writing-review & editing, Funding acquisition. **Pei-Wen Chu**: Formal analysis, Data curation, Visualization, Methodology, Investigation, Writing-review & editing. **Kuang-Kai Hsueh**: Methodology, Investigation, Writing-review & editing, Funding acquisition. **Shau-Kwaun Chen**: Conceptualization, Investigation, Resources, Writing-review & editing, Supervision.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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