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Effect of nicotinamide mononucleotide on osteogenesis in MC3T3-E1 cells against inflammation-induced by lipopolysaccharide

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Objective: Nicotinamide mononucleotide (NMN) is extensively utilized as an anti-aging agent and possesses anti-inflammatory properties. Lipopolysaccharide (LPS) activates Toll-like receptor 4, a process modulated by intracellular signaling pathways such as the Wnt/β-catenin pathway. This study investigated the impact of NMN on osteogenesis in the presence of LPS.
Methods: To elucidate the role of NMN in osteogenesis in the context of Gram-negative bacterial infection after LPS treatment, we cultured a mouse pre-osteoblast cell line (MC3T3-E1) and subsequently incubated it with NMN and/or LPS. We then evaluated osteogenic activity by measuring alkaline phosphatase activity, assessing gene expression and protein levels, and performing Alizarin Red S staining and immunocytochemistry.
Results: MC3T3-E1 cells underwent successful differentiation into osteoblasts following treatment with osteogenic induction medium. LPS diminished features related to osteogenic differentiation, which were subsequently partially reversed by treatment with NMN. The restorative effects of NMN on LPS-exposed MC3T3-E1 cells were further substantiated by elucidating the role of Wnt/β-catenin signaling, as confirmed through immunocytochemistry.
Conclusion: This study showed that infection with Gram-negative bacteria disrupted the osteogenic differentiation of MC3T3-E1 cells. This adverse effect was partially reversed by administering a high-dose of NMN. Drawing on these results, we propose that NMN could serve as a viable therapeutic strategy to preserve bone homeostasis in elderly and immunocompromised patients.

Keywords: Lipopolysaccharides; Nicotinamide mononucleotide; Osteogenesis; Wnt signaling pathway

Introduction

Bone is a highly dynamic and active tissue that continuously undergoes self-regeneration through a process known as bone remodeling [1]. This process involves the replacement of old bone with new bone to maintain homeostasis, utilizing various cell types that originate from mesenchymal stem cells (MSCs) or hematopoietic progenitors [2]. Osteogenesis, the development and formation of bone, begins with the migration of MSCs, which proliferate and differentiate into pre-osteoblasts, the precursors to osteoblasts [1,3]. Osteoblasts promote bone formation by secreting extracellular matrix proteins and by expressing key transcription factors, including Runt-related transcription factor 2 (Runx2) and Sp7 transcription factor (Sp7) [4-6]. These transcription factors are crucial for bone development, as they regulate the expression of various genes that code for extracellular

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matrix proteins, such as Col1a1, Col1a2, bone gamma-carboxyglutamate protein (Bglap), and alkaline phosphatase (Alp) [7,8]. However, disruptions in bone homeostasis can lead to bone-related disorders, with osteoporosis being a prime example. Osteoporosis increases the risk of fractures by diminishing bone mass, bone quality, and the maintenance of bone microarchitecture [9,10]. A previous study estimated the prevalence of osteoporosis among individuals aged 50 years and older in the European Union to be 6.6% in men and 22.1% in women in 2010 [11]. Clinically, there are two types of osteoporosis: (1) type I postmenopausal osteoporosis, which is caused by a deficiency in female hormones and results in an imbalance between bone formation and resorption [12,13]; and (2) type II osteoporosis, which occurs in the elderly and is associated with secondary hyperparathyroidism and an increase in bone marrow fat [14,15].

Nicotinamide mononucleotide (NMN) has recently been utilized as an anti-aging agent and serves as a vital precursor to nicotinamide adenine dinucleotide (NAD^+) [16,17]. Furthermore, NMN administration has been shown to increase intracellular NAD^+ levels [18]. NAD^+ plays an essential role as a coenzyme in numerous redox reactions, particularly in cellular metabolism [19]. Studies have shown that a reduction in NAD^+ levels leads to an accumulation of reactive oxygen species, which in turn causes oxidative stress and is linked to a decline in osteoblastogenesis [20,21]. Additionally, a prior study has found that elevated intracellular NAD^+ levels can protect against the detrimental effects of pro-inflammatory cytokines on islet function [22].

Lipopolysaccharide (LPS), a component of the cell wall in Gram-negative bacteria, is widely used to mimic Gram-negative bacterial infections in cell and animal models. Many studies have demonstrated that LPS can inhibit osteoblast differentiation in MC3T3-E1 cells [23] by intracellular signaling, including Wnt/β-catenin, p38, Smad, and other signal transduction pathways [24-27].

Previous studies have demonstrated that the Wnt/β-catenin signaling pathway plays a role in osteogenesis by promoting bone formation and inhibiting bone resorption [28,29]. In the canonical Wnt/β-catenin signaling pathway, Wnt ligands on the cell surface are activated by Wnt proteins, which in turn lead to the accumulation of β-catenin in the cytoplasm. This accumulation occurs because the activity of glycogen synthase kinase 3 (GSK3β; serine/threonine kinase), which normally degrades β-catenin, is inhibited. As β-catenin functions as a transcription factor, its translocation into the nucleus can initiate cell proliferation and differentiation [30]. A previous study reported that a reduction in β-catenin expression within chondrocytes resulted in the degeneration of articular cartilage typically seen in osteoarthritis [31]. Furthermore, Wnt1 has been identified as a regulator of both homeostasis and anabolic processes in the bone tissue of adult mice, particularly during bone formation induced by mechanical loading [32].

Therefore, this study aimed to investigate whether NMN protects against osteogenesis from inflammation caused by Gram-negative bacterial infections by regulating the Wnt/β-catenin signaling pathway.

Methods

1. Cell culture and differentiation

Pre-osteoblast MC3T3-E1 cells, obtained from the RIKEN Cell Bank in Tsukuba, Japan, served as the cell model for this study. These cells were cultured in alpha-minimum essential medium (Gibco) enriched with 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin (Lonza). To induce osteoblast differentiation, the cells were plated in 6-well plates and incubated for 24 hours. The next day, the medium was replaced with osteogenic induction medium (OIM) containing 10 mM β-glycerophosphate (Sigma-Aldrich) and 50 μg/mL ascorbic acid (Sigma-Aldrich). This OIM was refreshed every 3 days.

2. Cell viability assay

Cell viability was assessed to determine the cytotoxic dose of LPS (Sigma-Aldrich) and NMN (Genex Formulas). We evaluated the viability of the cells using the cell counting kit-8 (CCK-8; Dojindo). MC3T3-E1 cells were plated in 96-well plates at a density of 1×10^3 cells per well. Following incubation with varying concentrations of LPS or NMN, we added the CCK-8 reagent to each well and incubated the cells for 2 hours at 37 °C.

3. Evaluation of alkaline phosphatase activity

MC3T3-E1 cells were plated in 6-well plates at a density of 5×10^4 cells/well. On the subsequent day, the culture medium was replaced with OIM supplemented with LPS and/or NMN. After a period of 7 days, the cells were collected, and total protein was isolated using radioimmunoprecipitation assay (RIPA) buffer (Thermo Fisher Scientific) enhanced with a protease inhibitor cocktail (Thermo Fisher Scientific). ALP activity was assessed with an ALP assay kit (Abcam), following the protocol provided by the manufacturer. For the ALP assay, a consistent quantity of protein (60 μg) was utilized across all samples. The total protein concentration for each sample was ascertained using a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific).

4. Intracellular mineralization analysis by Alizarin Red S staining

MC3T3-E1 cells were plated in 6-well plates at a density of 1.25×10^4 cells per well, and the following day, the culture medium was replaced with OIM containing LPS and/or NMN to induce osteoblast differentiation over a period of 14 days. Subsequently, the medium was discarded, and the cells were rinsed with filtered Dulbec-

co's phosphate-buffered saline (DPBS; Biowest). The cells were then fixed with 4% paraformaldehyde at 25 ℃ for 1 hour. After removing the fixative, each well was washed three times for 5 minutes with distilled water. Alizarin Red S solution (40 mM; Sigma-Aldrich) was applied to each well, and the plates were incubated at 25 ℃ for 30 minutes. The staining solution was then discarded, and each well was washed three times for 5 minutes with distilled water. Optical assessment of the stained cells was performed using an Eclipse 80i microscope (Nikon). For quantitative analysis, the Alizarin Red S-stained cells were destained with 1 mL of 0.1 N NaOH per well at 25 ℃ for 25 minutes. The resulting solution was collected, and its absorbance was measured at a wavelength of 540 nm.

5. Reverse transcription-quantitative polymerase chain reaction

Cells were seeded in 12-well plates at a density of 5×10^4 cells/well and treated with OIM containing LPS or NMN the following day. The cells were harvested after 3 days, and total RNA was extracted using the TRIzol reagent (Invitrogen), according to the manufacturer's instructions. RNA (1 μg) was reverse transcribed into cDNA using a cDNA synthesis kit (Takara Bio). Real-time polymerase chain reaction (PCR) was performed using SYBR Green PCR Master Mix (Bioline) on the QuantStudio 1 Real-Time PCR System (Thermo Fisher Scientific). Primer sequences used in this study are listed in Table 1. The PCR protocol consisted of an initial denaturation at 95 ℃ for 10 minutes, followed by 40 cycles of denaturation at 95 ℃ for 10 seconds, annealing at the optimal temperature for each primer for 15 seconds, and extension at 72 ℃ for 30 seconds.

6. Western blot analysis

To quantify the amount of intracellular protein, we performed Western blot analysis using total protein extracted from MC3T3-E1 cells. The extraction of total protein was carried out according to the method previously described for the ALP activity assay. We separated equal quantities of protein (20 μg) from each sample by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subsequently transferred them onto polyvinylidene fluoride membranes. The membranes were blocked with 5% skim milk at 25 ℃ for 2 hours, followed by overnight incubation at 4 ℃ with primary antibodies against ALP (1:200), β-catenin (1:200), and glyceraldehyde 3-phosphate dehydrogenase (1:5,000). These primary antibodies were sourced from Santa Cruz Biotechnology. After washing the membranes three times with tris-buffered saline containing 1% Tween 20, they were incubated with an anti-mouse horseradish peroxidase-conjugated immunoglobulin G (IgG) secondary antibody (Cell Signaling Technology) for 2 hours at 25 ℃. We then analyzed the immunoreactive proteins on the membranes using an iBright CL750 Imaging System (Invitrogen). The data were processed using ImageJ software (National Institutes of Health).

7. Immunocytochemistry

To investigate the involvement of the Wnt/β-catenin signaling pathway in this study, we confirmed β-catenin translocation using immunocytochemistry. MC3T3-E1 cells were plated at a density of 2.5×10^3 cells per well in an 8-well chamber slide. The following day, these cells were treated with OIM containing LPS and/or NMN for 4 hours. Cells were then fixed with 4% paraformaldehyde at 25 ℃ for 30 minutes and permeabilized with 0.1% Triton X-100 in DPBS. After blocking with 5% bovine serum albumin in DPBS for 1 hour at 25 ℃, the cells were incubated overnight at 4 ℃ with a primary antibody against β-catenin (1:200; Santa Cruz Biotechnology). The following steps were performed using the Alexa Fluor 488 Tyramide SuperBoost Kit, goat anti-mouse IgG (Invitrogen), in accordance with the manufacturer's instructions. The cells were then counterstained and mounted using VECTASHIELD Antifade Mounting Medium with 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories). The stained cells were visualized and analyzed with a Nikon Eclipse 80i microscope.

8. Statistical analysis

The experiments conducted in this study were replicated a minimum of three times. The data are reported as the mean±standard error of the mean. For statistical analysis, we utilized GraphPad Prism 5.01 software (GraphPad Software). We compared various groups using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. A *p*-value of less than 0.05 was considered to indicate statistical significance.

Results

1. Changes in MC3T3-E1 cells during osteogenic differentiation

MC3T3-E1 cells were cultured in OIM to induce osteogenic differ-

Table 1. Primer sequences for quantitative real-time polymerase chain reaction

Gene name		Sequences (5′→3′)
Alp1	Forward	CCA ACT CTT TTG TGC CAG AGA
	Reverse	TGA CAT TCT TGG CTA CAT TGG TG
Bglap	Forward	TAG TGA ACA GAC TCC GGC GCT A
	Reverse	TGT AGG CGG TCT TCA AGC CAT
Runx2	Forward	ACT CTT CTG GAG CCG TTT ATG
	Reverse	GTG AAT CTG GCC ATG TTT GTG
Sp7	Forward	ATG GCG TCC TCT CTG CTT G
	Reverse	TGA AAG GTC AGC GTA TGG CTT
Gapdh	Forward	TGA CCA CAG TCC ATG CCA TC
	Reverse	GAC GGA CAC ATT GGG GGT AG

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entiation. To identify whether differentiation had occurred, the mRNA expression of markers (actin-like protein 1 [*Alp1*], *Bglap*, *Runx2*, *Sp7*) related to osteogenesis was assessed by quantitative real-time PCR (qRT-PCR). We found that the mRNA expression levels of the markers were higher in the induced cells than in their non-induced counterparts on day 3 of differentiation (Figure 1A). To assess ALP activity in the differentiated MC3T3-E1 cells, we treated the cells with OIM for 3 and 7 days. We observed a significant increase in ALP activity in the OIM-treated group after 7 days, whereas there was no significant difference in ALP activity between the non-treated and OIM-treated groups at the 3-day mark (Figure 1B). Furthermore, the current study confirmed successful osteogenic differentiation following OIM treatment, as evidenced by Alizarin Red S staining (Figure 1C). Intracellular mineralization was found to increase in a time-dependent manner throughout the 14-day period.

2. LPS can inhibit the osteogenic differentiation of MC3T3-E1 cells

At each tested concentration of LPS (0.01, 0.1, and 1 μg/mL), the CCK-8 assay revealed no cytotoxic effects in MC3T3-E1 cells (Figure 2A).

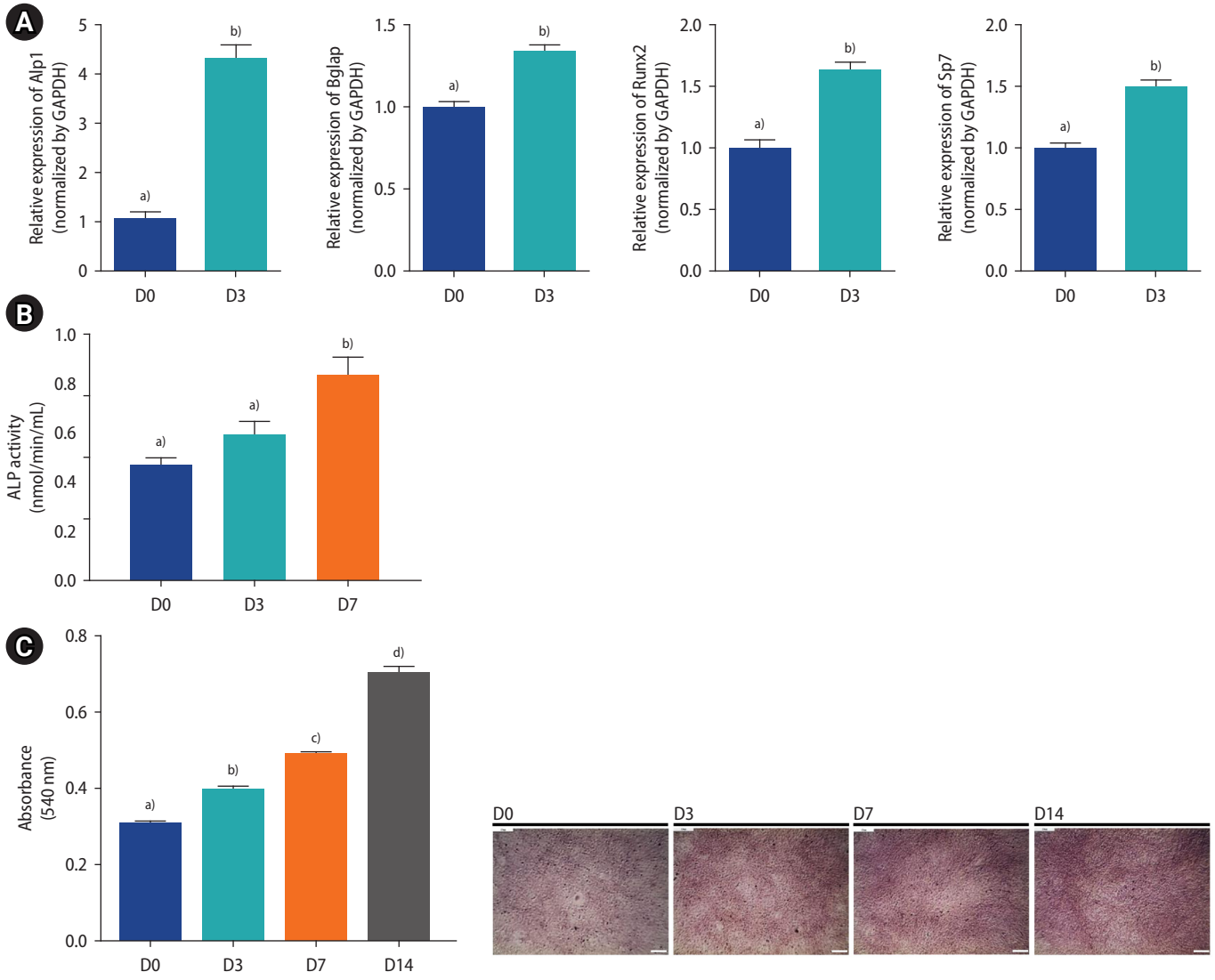

Figure 1. Induction of osteogenic differentiation in MC3T3-E1 cells. MC3T3-E1 pre-osteoblast cells were cultured in osteogenic induction medium for durations of 3, 7, or 14 days. (A) The mRNA expression levels of actin-like protein 1 (*Alp1*), bone gamma-carboxyglutamate protein (*Bglap*), Runt-related transcription factor 2 (*Runx2*), and *Sp7* transcription factor (*Sp7*) were evaluated using quantitative real-time polymerase chain reaction, with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) serving as the internal control. (B) Alkaline phosphatase (ALP) activity was quantified in relation to the total protein content of the MC3T3-E1 cells. (C) Alizarin Red S staining facilitated the visualization of mineralized nodules. All images were captured at ×40 magnification, and a scale bar, presented in white, indicates 200 μm. [a),b),c),d)]Distinct letters denote statistically significant differences ($p<0.05$).

Moreover, osteogenic differentiation induced by LPS treatment resulted in a dose-dependent decrease in the mRNA markers of osteogenesis, as measured by RT-qPCR. Notably, mRNA expression levels remained unchanged at the lowest LPS concentration (0.01 μg/mL), whereas the highest concentration (1 μg/mL) significantly reduced the expression levels of *Alp1*, *Bglap*, *Runx2*, and *Sp7* (Figure 2B). Additionally, LPS markedly suppressed ALP activity and intracellular mineralization (Figure 2C, 2D). In summary, an LPS concentration of 1 μg/mL disrupted osteogenesis in the MC3T3-E1 pre-osteoblast cell line.

3. NMN showed different cell activities according to its concentration

NMN promoted proliferation in a dose-dependent manner. The

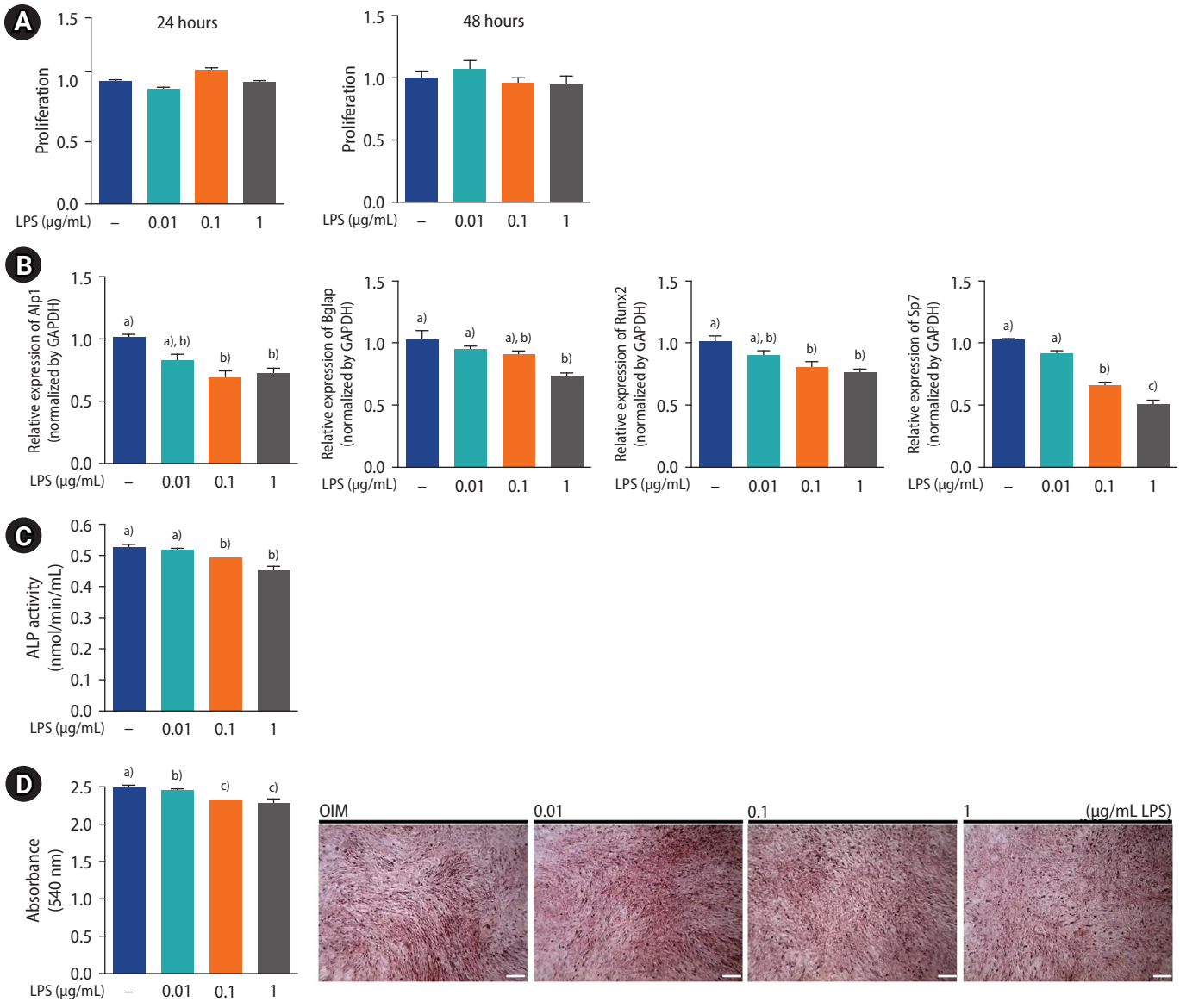

Figure 2. Effect of lipopolysaccharide (LPS) on proliferation and osteogenic differentiation in MC3T3-E1 cells. (A) Cell proliferation was assessed using the cell counting kit-8 assay. (B, C) MC3T3-E1 cells were cultured in osteogenic induction medium and treated with LPS at concentrations of 0.01, 0.1, and 1 μg/mL. (B) The mRNA expression levels of actin-like protein 1 (*Alp1*), bone gamma-carboxyglutamate protein (*Bglap*), Runt-related transcription factor 2 (*Runx2*), and Sp7 transcription factor (*Sp7*) were evaluated using quantitative real-time polymerase chain reaction, with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) serving as the internal control. Additionally, (C) alkaline phosphatase (ALP) activity and (D) mineralization were quantified. All images were captured at ×40 magnification, and a scale bar is presented in white at the bottom of each image (200 μm). OIM, osteogenic induction medium. [a),b),c)]Distinct letters indicate statistically significant differences ($p<0.05$).

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expression of genes related to osteogenic differentiation did not change compared to the control group at 0.1 and 1 mM NMN, but those markers decreased at higher NMN doses. Consistent with the qRT-PCR findings, ALP activity and intracellular mineralization at NMN concentrations of 0.1 and 1 mM were similar to those observed in the control group (Figure 3). At an NMN concentration of 1 mM, the proliferation of MC3T3-E1 cells was augmented without any observed inhibitory effects during osteoblastic differentiation. Consequently, we selected 1 mM NMN to investigate its potential to mitigate LPS-induced inflammation.

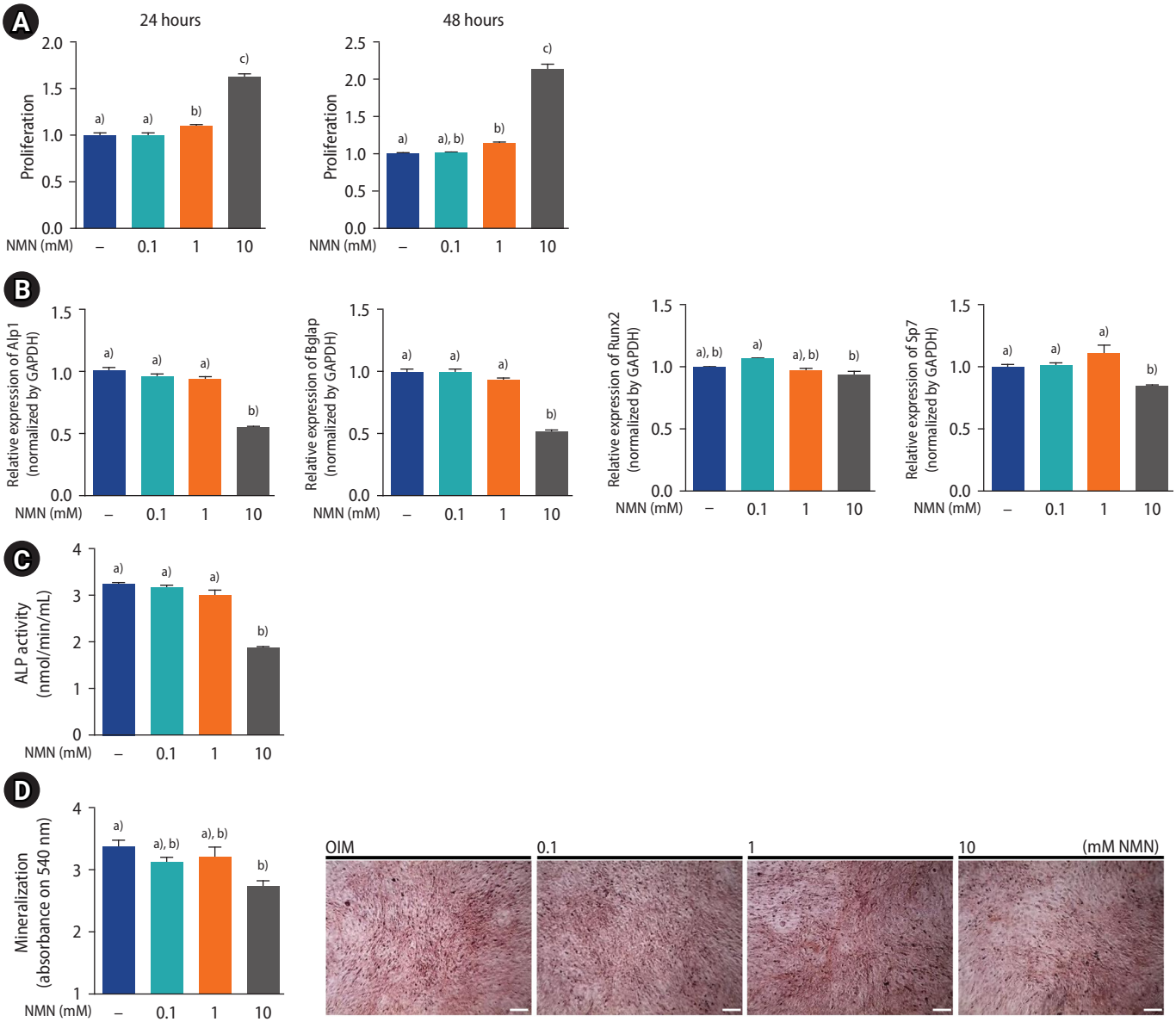

Figure 3. Effect of nicotinamide mononucleotide (NMN) on proliferation and osteogenic differentiation in MC3T3-E1 cells. (A) A cell counting kit-8 assay was conducted in MC3T3-E1 treated with NMN for 24 or 48 hours. (B, C) MC3T3-E1 cells were cultured in osteogenic induction medium and treated with NMN at 0.1, 1, and 10 mM. (B) The mRNA expression levels of actin-like protein 1 (*Alp1*), bone gamma-carboxyglutamate protein (*Bglap*), Runt-related transcription factor 2 (*Runx2*), and Sp7 transcription factor (*Sp7*) were evaluated using quantitative real-time polymerase chain reaction, with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) serving as the internal control. (C) Alkaline phosphatase (ALP) activity was measured by using total protein as a sample. (D) Mineralization was assessed using Alizarin Red S staining. All images were captured at ×40 magnification, and a scale bar is presented in white at the bottom of each image (200 μm). OIM, osteogenic induction medium. [a),b),c)]Distinct letters indicate statistically significant differences ($p<0.05$).

4. Osteogenic differentiation interrupted by LPS was partially restored with NMN treatment

NMN and LPS were administered simultaneously to MC3T3-E1 cells during the induction of osteogenic differentiation to determine if NMN could mitigate the inflammation-induced disruption of differentiation caused by LPS. The mRNA levels of *Alp1*, *Bglap*, and *Sp7* in cells that received both NMN and LPS were higher than those in cells treated solely with LPS (Figure 4A). The ALP activity in the group receiving the combined treatment was comparable to that observed in the control and LPS-only treatment groups; however, the quantity of ALP in the co-treatment group was significantly greater (Figure 4B, 4C). Additionally, intracellular mineralization was restored when NMN and LPS were administered together (Figure 4D). In summary, 1 mM NMN appeared to partially counteract the LPS-induced inhibition of osteogenic differentiation.

5. NMN can improve the osteogenic differentiation hindered by LPS through the Wnt/β-catenin signaling pathway

To investigate the involvement of the Wnt/β-catenin signaling pathway in the effects of NMN, we analyzed the expression pattern

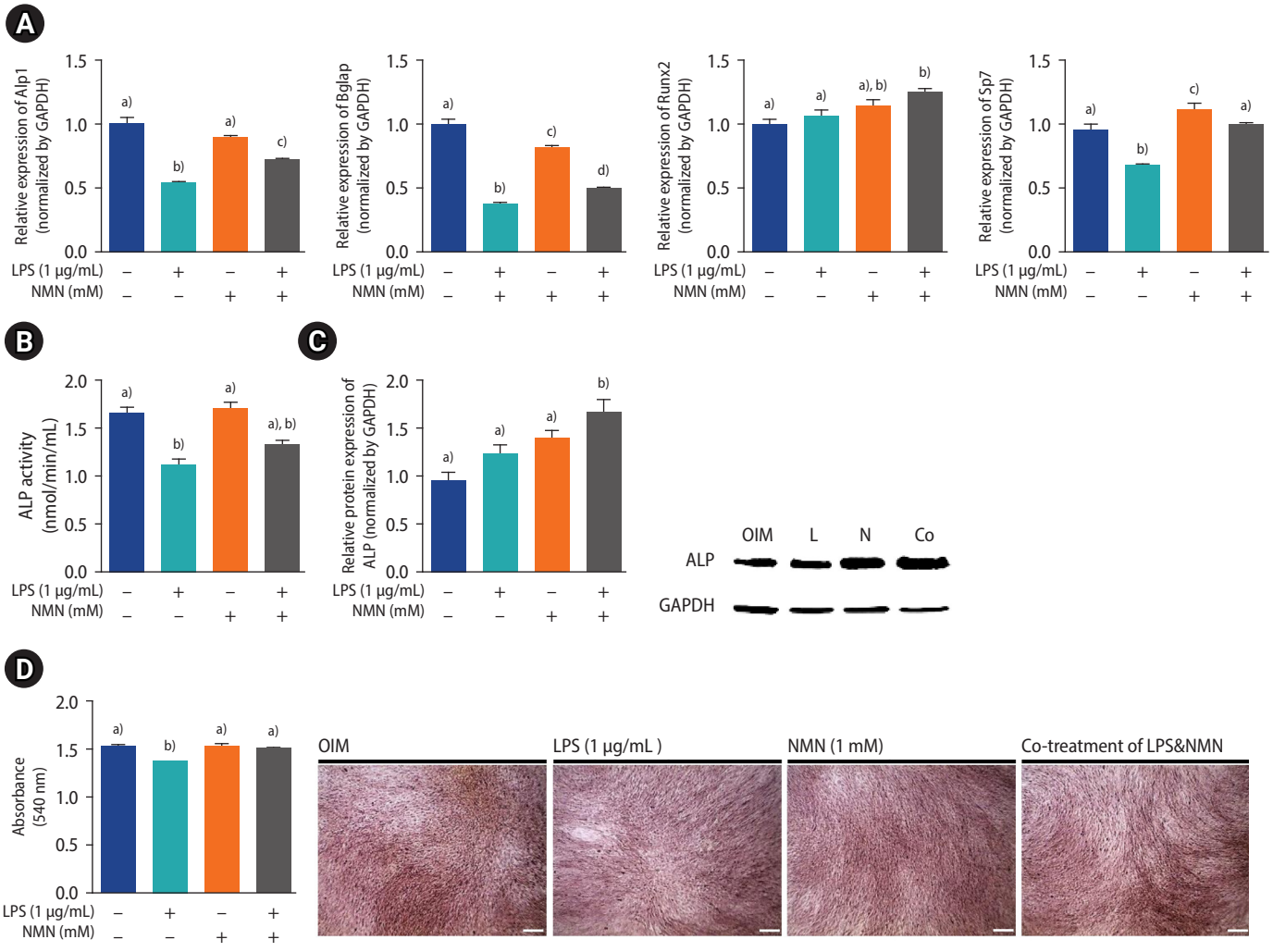

Figure 4. Protective effect of nicotinamide mononucleotide (NMN) on osteogenic differentiation in MC3T3-E1 cells treated with lipopolysaccharide (LPS). MC3T3-E1 cells were treated with LPS and/or NMN during osteogenesis. (A) The mRNA expression of osteogenesis markers was measured using quantitative real-time polymerase chain reaction. (B) Alkaline phosphatase (ALP) activity and (C) the total amount of ALP in the total protein of MC3T3-E1 cells. (D) Mineralization of MC3T3-E1 cells was assessed by Alizarin Red S staining. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. All images were captured at ×40 magnification, and a scale bar is presented in white at the bottom of each image (200 μm). *Alp1*, actin-like protein 1; *Bglap*, bone gamma-carboxyglutamate protein; *Runx2*, Runt-related transcription factor; *Sp7*, Sp7 transcription factor; OIM, osteogenic induction medium; L, LPS; N, NMN; Co, co-treatment of LPS&NMN. [a),b),c),d)]Distinct letters indicate statistically significant differences ($p<0.05$).

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of β-catenin. We observed the translocation of β-catenin by performing immunocytochemistry. In the group treated with LPS, β-catenin was localized in the cytoplasm rather than the nucleus. In contrast, nuclear translocation of β-catenin was evident in both the NMN-treated and co-treated groups, as indicated by the red arrowhead. Furthermore, there was a significant difference in the fluorescence intensity of the nucleus between the LPS-treated group and the other groups (Figure 5A). However, the difference in the levels of β-catenin between the groups was not discernible (Figure 5B).

Discussion

In this study, MC3T3-E1 cells, also known as mouse pre-osteoblasts, served as an *in vitro* model for osteogenesis. This choice was informed by previous research indicating that MC3T3-E1 cells express osteogenesis-related biomarkers, including ALP, BGLAP, RUNX2, and SP7, during their differentiation process [33,34]. We identified an increase in osteogenic markers and intracellular mineralization in osteogenesis-induced MC3T3-E1 cells. When these cells were treated with LPS during osteogenesis, their mRNA expression levels (*Alp1*, *Bglap*, *Runx2*, and *Sp7*) and ALP activity declined in a dose-dependent manner, and mineralization was inhibited by high-dose LPS (1 μg/mL). NMN at a concentration of 10 mM was found to promote MC3T3-E1 cell proliferation, but also led to decreased mRNA expression levels, ALP activity, and mineralization. We observed that the pH of the medium changed during the experiments, increasing with the concentration of NMN. This suggests that the inhibitory effects of 10 mM NMN treatment may be due to the altered pH levels in the medium. Conversely, a lower concentration of NMN (1 mM) stimulated cell proliferation without adversely affecting osteogenic differentiation. Consequently, we investigated whether 1 mM NMN could mitigate the disruption of osteogenesis-induced by LPS. Co-treatment with LPS and NMN resulted in a partial recovery of osteogenesis-related mRNA markers that had been affected by LPS. Notably, the levels of *Runx2* and *Sp7*, which are transcription factors, were elevated and restored with NMN treatment. Additionally, NMN demonstrated protective effects on ALP activity and mineralization. To explore the potential involvement of the Wnt/β-catenin signaling pathway in NMN's effect on osteogenesis, we examined the intracellular patterns and expression of β-catenin. While there was no significant difference in the total amount of β-catenin among the groups, we observed that β-catenin translocated to the nucleus upon NMN treatment, similar to what occurred in response to OIM treatment. Furthermore, the fluorescence intensity of β-catenin was also restored with NMN treatment. In summary, our results suggest that NMN has a protective role against LPS-induced osteogenesis disruption, potentially through the Wnt/β-catenin signaling pathway.

NMN is a crucial intermediate in the biosynthesis of NAD^+, whose levels diminish with aging [16]. In previous studies, the administration of NMN improved intracellular NAD^+ levels, suggesting that it could be used to treat age-related diseases such as osteoporosis, type 2 diabetes, ischemia-reperfusion injury, and Alzheimer's disease [35,36]. In addition, Huang and Tao [37] reported that NMN diminished osteogenic inhibition induced by glucocorticoids, a category of anti-inflammatory drugs, by regulating the sirtuin 1 (SIRT1)/peroxisome proliferator-activated receptor-γ coactivator 1-α (PGC-1α) signaling pathway in bone MSCs. Lu et al. [38] also found that NMN relieved senescence in osteoblasts and improved bone repair in osteoporotic mice. The Wnt/β-catenin signaling pathway has been shown to stimulate bone formation and remodeling during osteogenesis [29], and various studies have demonstrated that osteogenic differentiation was induced in MC3T3-E1 cells via the Wnt/β-catenin signaling pathway [33,39,40]. However, to date, no research has clarified whether NMN can ameliorate LPS-induced impairment of osteogenesis or if NMN's influence on osteogenesis is mediated by the Wnt/β-catenin signaling pathway. In our study, we have shown that NMN offers partial protection against the LPS-induced suppression of osteogenesis. Additionally, NMN treatment enhanced the nuclear translocation of β-catenin, which functions as a transcription factor. These findings indicate that NMN protects against LPS-induced osteogenic decline by modulating the Wnt/β-catenin signaling pathway.

A previous study demonstrated that NMN alleviated the loss of bone induced by aluminum, a persistent environmental contaminant, by inhibiting the thioredoxin-interacting protein (TXNIP)-NOD-like receptor family pyrin domain-containing protein 3 (NLRP3) inflammasome in MC3T3-E1 cells [41]. Although that experiment revealed that NMN had an alleviating effect on bone loss in a dose-dependent manner from 0.1 to 10 mM, our results showed that high-dose NMN (10 mM) had an adverse effect on osteogenesis. Antioxidants, such as NMN, exert complex effects on cell proliferation and differentiation depending on the pH due to shifts in pKa values reflecting alterations in ionized hydroxyl groups or other functional groups within phenolic compounds [42]. Therefore, the observed detrimental effects on osteogenesis at high NMN doses may be attributed to pH changes in the medium. Furthermore, our study revealed that NMN could enhance osteogenesis that was otherwise impaired, by modulating the Wnt/β-catenin signaling pathway, although it did not completely counteract the impairment. This is because osteogenic differentiation in MC3T3-E1 cells can also proceed via other pathways that regulate differentiation, such as the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway [43]. Our study had limitations, including the use of a mouse cell line rather than a human cell line or primary cells, and it did not confirm whether NMN

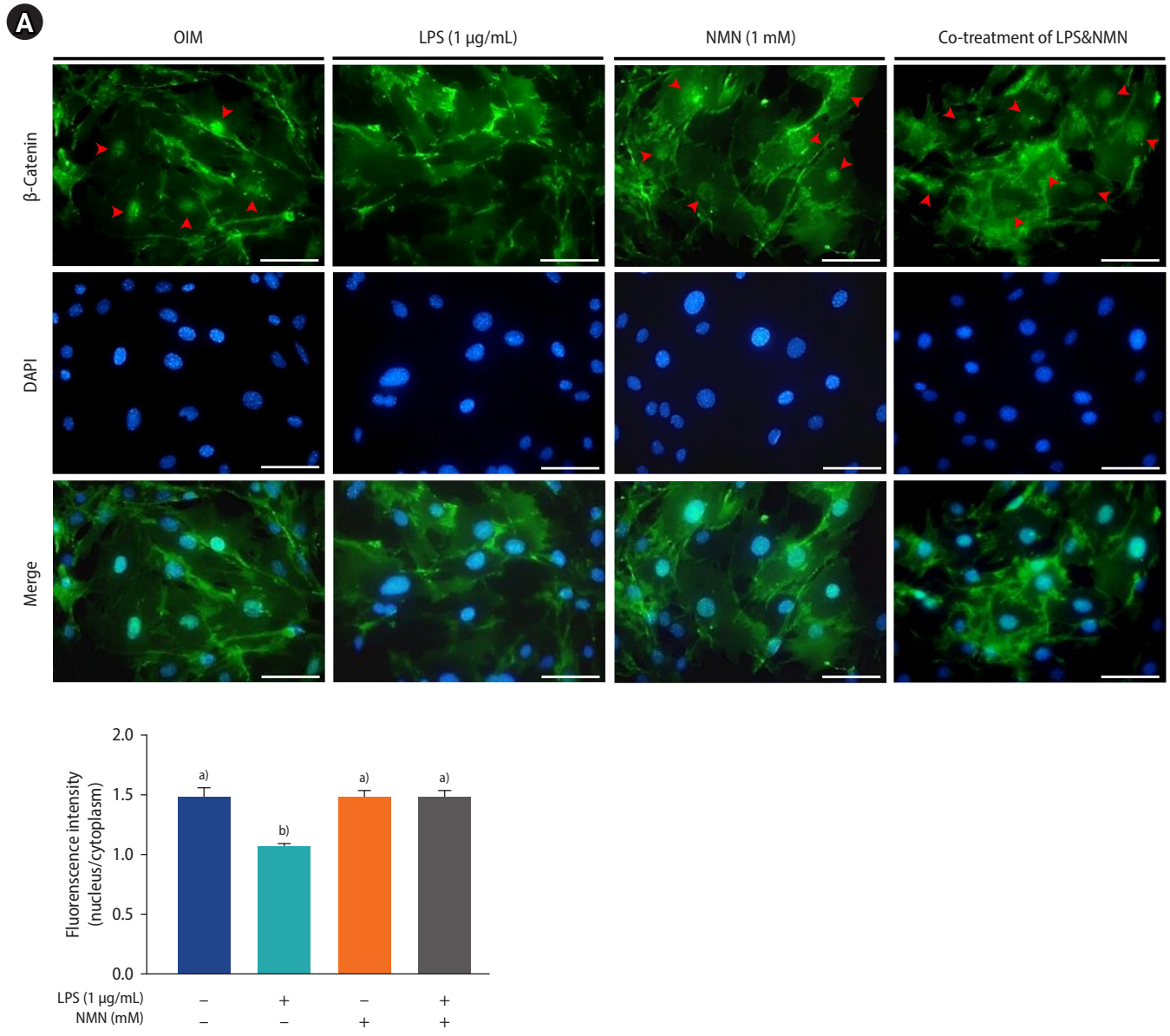

Figure 5. Expression of β-catenin at MC3T3-E1 cells during osteogenic differentiation. To determine whether the Wnt/β-catenin signaling pathway was involved in this experiment, immunocytochemistry and Western blotting were conducted. (A) Immunocytochemistry was performed using β-catenin monoclonal antibody coupled with Alexa 488 conjugated secondary antibodies (green). There were red arrowheads to point to the presence of translocation into nucleus. 4′,6-Diamidino-2-phenylindole (DAPI) was used for nuclear counterstaining (blue). Fluorescence images were obtained using a Nikon Eclipse 80i microscope at ×400 magnification (scale bar=50 μm). Fluorescence intensity was evaluated using ImageJ software (National Institutes of Health). (B) β-Catenin levels were measured using Western blot analysis. Glyceraldehyde 3-phosphate dehydrogenase (Gapdh) was used as an internal control. OIM, osteogenic induction medium; LPS, lipopolysaccharide; NMN, nicotinamide mononucleotide; L, LPS; N, NMN; Co, co-treatment of LPS&NMN. [a),b)]Distinct letters indicate statistically significant differences (p<0.05).

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increased the intracellular NAD^+ levels in MC3T3-E1 cells. However, NMN's ability to restore intracellular NAD^+ levels in these cells has been documented in previous research [41]. Additionally, we did not ascertain whether other molecules associated with the Wnt/β-catenin signaling pathway, such as Wnt and GSK3β, were elevated or activated (phosphorylated). In future studies, we plan to explore additional signaling pathways that regulate osteogenesis.

In summary, our study showed that LPS inhibits osteogenic differentiation of pre-osteoblasts by diminishing the expression of mRNA markers, ALP activity, and mineralization, and that NMN alleviated LPS-induced osteogenic inhibition by regulating the Wnt/β-catenin signaling pathway. Notably, this study is the inaugural work to show that NMN's protective effects against inflammation in osteogenesis are mediated by the Wnt/β-catenin signaling pathway. Based on our findings, we propose that NMN holds promise as a therapeutic agent for the treatment of age-related and immunocompromised patients.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

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Author contributions

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