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Original Article

Myeloid differentiation factor 2 inhibitors exert protective effects on lipopolysaccharides-treated human dental pulp cells via suppression of toll-like receptor 4-mediated signaling

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Abstract *Background/purpose:* The toll-like receptor 4 (TLR4)-myeloid differentiation factor 2 (MD-2) complex is known to have a role in inflammation. Blocking MD-2 can suppress inflammatory process. However, the actual action of MD-2 inhibitors, including MAC28, L6H21, and 2i-10, on the inflamed human dental pulp cells (HDPCs) has never been examined. This study aims to determine the pharmacological effects of these 3 compounds on cell viability, inflammation, and osteo/odontogenic differentiation of lipopolysaccharide (LPS)-treated HDPCs.
Materials and methods: HDPCs were pretreated with 10 μM of MAC28, L6H21, or 2i-10 for 2 h followed by either 20 μg/mL LPS or vehicle for 24 h. Cell viability was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The mRNA and expression of the proteins TLR4, MD-2, tumor necrosis factor alpha (TNF-α), and interleukin 6 (IL-6) were determined using quantitative real-time polymerase chain reaction (qRT-PCR) and Western

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blot analysis. Osteo/odontogenic differentiation was investigated using qRT-PCR and Alizarin Red staining.
Results: LPS did not alter cell viability but significantly increased the expression levels of TLR4, MD-2, TNF-α, and IL-6 in HDPCs while the osteo/odontogenic differentiation ability decreased significantly when compared to the vehicle-treated group. MAC28, L6H21, and 2i-10-pretreatment in LPS-treated HDPCs reduced inflammation and restored osteo/odontogenic differentiation to similar levels as the vehicle-treated group.
Conclusion: MAC28, L6H21, and 2i-10 exhibited equal efficacy in attenuating inflammation through downregulation of TLR4-MD-2 signaling and restored osteo/odontogenic differentiation in LPS-treated HDPCs. These MD-2 inhibitors could be considered as the potential therapeutic supplement for curing inflammation of dental pulp in future studies.

Introduction

Myeloid differentiation factor 2 (MD-2) is a protein which is fundamental to the activation of toll-like receptor 4 (TLR4) signaling which plays a critical role in the inflammatory process in different tissues.[1—3] Several studies have verified that the TLR4-MD-2 complex presents on extracellular surfaces, including the cell membranes of dentinal-pulpal cells.[4—6] Lipopolysaccharide (LPS) is one of the most important ligands for the activation of TLR4-MD-2 signaling by accommodating the hydrophobic binding site of MD-2, resulting in inflammation.[2] Recent studies have explored the use of pharmacological interventions of MD-2 blockers in the inhibition of TLR4 signaling which showed beneficial effects on inflamed tissues.[2,7] Pulpitis is an inflammation of dental pulp, resulting in toothache, which is the most common complaint in dental practice.[8,9] The blocking of TLR4 signaling via MD-2 inhibitors is a potential curative approach for pulpitis.

Over the past ten years, MD-2 inhibitors have been developed from natural and synthetic compounds. MAC28, a compound derived from curcumin, has been shown to block TLR4 signaling in LPS-stimulated macrophages by binding directly to MD-2.[10] L6H21, a chalcone-derivative, reduced inflammation in LPS-treated macrophages by binding to MD-2.[1] One novel MD-2 blocker 2i, a cinnamamide derivative compound, attenuated inflammation by blocking MD-2 activity.[11] Recently, a new cinnamamide derivative, 2i-10, has been developed, but the pharmacological action of 2i-10 in LPS-treated cells, particularly in human dental pulp cells (HDPCs), has never been investigated. Furthermore, details regarding the pharmacological impact of MAC28 and L6H21 in LPS-treated HDPCs are still unavailable.

This study proposes to determine the pharmacological actions of MAC28, L6H21, and 2i-10 on cell viability, inflammation, and osteo/odontogenic differentiation of LPS-treated HDPCs. We hypothesized these compounds would attenuate inflammation and restore differentiation in LPS-treated HDPCs.

Materials and methods

Dental pulp tissue collection and ethical approval

This study was conducted following the authorization from the Human Experimentation Committee of the Faculty of Dentistry, Chiang Mai University, Thailand (ethical approval number: 44/2020). After obtaining informed consent, healthy pulp tissues were collected from subjects (aged 19—21 years old) scheduled for tooth extraction for orthodontic purposes.

Cell culture

Isolation of HDPCs from subjects was carried out under the procedure reported by Vaseenon and colleagues.[12] The HDPCs were cultured in α-MEM (Sigma—Aldrich, St Louis, MO, USA), enriched with 10% fetal bovine serum (FBS) (Life Technologies, Carlsbad, CA, USA), 100 U/mL of penicillin and 100 μg/mL of streptomycin (Sigma—Aldrich), and 100 μmol/L of L-ascorbic acid (Sigma—Aldrich) at 37 °C in an atmosphere of 5% CO_2 in the air. The medium was replenished every other day. HDPCs from passages two to four were used in the experiment. A mixed population of HDPCs was used to resemble the diverse nature of the dental pulp. In each experiment, the cells from each donor were triplicated and measured for each parameter thrice, and finally calculated the average value of each donor from these three measurements for data analysis. The characterization of primary HDPCs and stem cell marker (CD81) was confirmed by inverted-light microscopy and flow cytometry as shown in Fig. 1.

The experimental protocol

HDPCs were divided into 9 groups, n = 8/group,[12] as follows: 1) control: incubated with α-MEM; 2) vehicle group: incubated with 0.0001% dimethyl sulfoxide (DMSO); 3) LPS group: incubated with 20 μg/mL LPS; 4) MAC28 group:

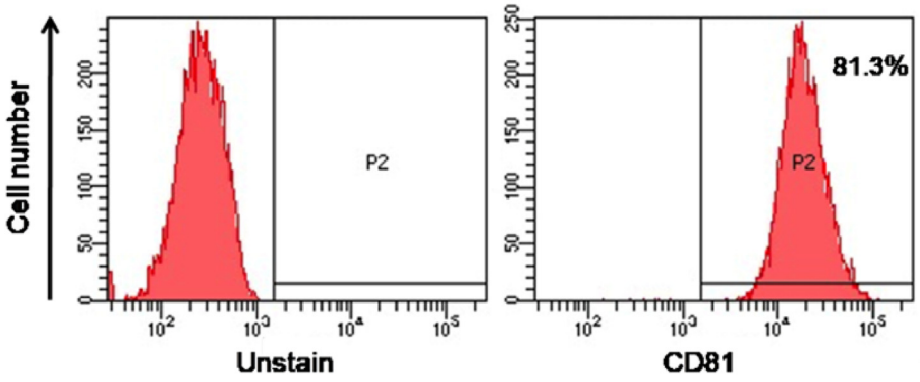

Figure 1 Illustration of a flow cytometry plot of HDPCs against CD81. The number on the right graph indicates the percentage of CD81 positive cells in dental pulp cell populations.

incubated with 10 μM MAC28; 5) LPS + MAC28 group: pretreated with 10 μM MAC28 for 2 h, and then incubated with 20 μg/mL LPS; 6) L6H21 group: incubated with 10 μM L6H21; 7) LPS + L6H21 group: pretreated with 10 μM L6H21 for 2 h, and then incubated with 20 μg/mL LPS; 8) 2i-10 group: incubated with 10 μM 2i-10; and 9) LPS + 2i-10 group: pretreated with 10 μM 2i-10 for 2 h, and then incubated with 20 μg/mL LPS. After 24 h incubation, cell viability, inflammation, and differentiation were assessed as described in the next sections. The diagram of this study protocol is summarized in Fig. 2.

Preparation of LPS, MAC28, L6H21, and 2i-10

Twenty μg/mL of LPS, and 10 μM of MAC28, L6H21, and 2i-10 were used in the experiments, concentrations based on previous studies.[1,10–13] LPS from *Escherichia coli* (0111: B4; #0000110081) were bought from Sigma–Aldrich. MAC28, L6H21, and 2i-10 were supplied by Wenzhou Medical University in Zhejiang, China. The 1 M concentration of MAC28 (mw = 469.53), L6H21 (mw = 298.34), and 2i-10 (mw = 526.23) were firstly prepared by liquefying 4.695 mg of MAC28, 2.983 mg of L6H21, and 5.262 mg of 2i-10 in 10 mL of 100% DMSO. The solutions were subsequently mixed with α-MEM to a dilution of 10 μM for cell treatment. The final concentration of soluble DMSO in α-MEM containing MAC28, L6H21, and 2i-10 groups was 0.0001%. Therefore, we used 0.0001% DMSO for vehicle controls.

Determination of cell viability

A density of 10^4 cells/well HDPCs was seeded in 96-well plates. After culturing in α-MEM, supplemented with 10% FBS and 1% penicillin-streptomycin at 37 °C and a 5% CO_2 environment for 24 h, HDPCs were treated in the assigned media for another 24 h. Cell viability was determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Invitrogen, Waltham, MA, USA) assay. Fifteen microliters of the MTT stock solution (12 mM) were added and incubated at 37 °C for 4 h. These were subsequently dissolved in 100 μL of DMSO following the protocol described by Prathumsap and colleagues.[14] The microplate reader (BioTek, Winooski, VT, USA) was set at 540 nm for absorbance reading. The absorbance in the control group was considered 100% viability.

Determination of inflammatory markers by quantitative real-time polymerase chain reaction (qRT-PCR)

To determine the inflammatory markers, HDPCs, in 6-well plates at a density of 2.5×10^5 cells/well, were allocated to 9 experimental groups following the study protocol. After 24-h of treatment, qRT-PCR was performed following the protocol described by Ongnok and colleagues.[15] A qRT-PCR assay by the Bio-Rad Cx96 Detection System (Bio-Rad Laboratories Ltd., Hercules, CA, USA) was used to analyze gene expressions. Each mRNA was quantified by standardization with the housekeeping enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and quantified using the average threshold cycle (C_T) method. The C_T of target mRNAs and the C_T of GAPDH were calculated using the equation $2^{-\Delta\Delta C_T}$ where $\Delta\Delta C_T = (C_{T,\ target} - C_{T,\ GAPDH})_{experimental\ group} - (C_{T,\ target} - C_{T,\ GAPDH})_{control}$.[16] The primer pairs for TLR4, MD-2, tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6), and GAPDH employed in this study are displayed in Table 1.

Determination of inflammatory markers by Western blot analysis

HDPCs (2.5×10^5 cells/well) were seeded in 6-well plates. After 24-h of treatment, the expression levels of TLR4, MD-2, TNF-α, and IL-6 proteins were calculated by Western blot analysis. The concentration of supernatant protein was quantified, loaded, and proceeded with gel electrophoresis before being transferred onto nitrocellulose membranes, as narrated in a previous study.[7] The subsequent primary antibodies at 1:1000 dilution were used to investigate the immunoblots: anti-TLR4 (rabbit; Abcam, Cambridge, UK; ab13556), anti-MD-2 (rabbit; Abcam; ab24182), anti-TNF alpha (rabbit; Abcam; ab9635), anti-IL-6 (mouse; Abcam;

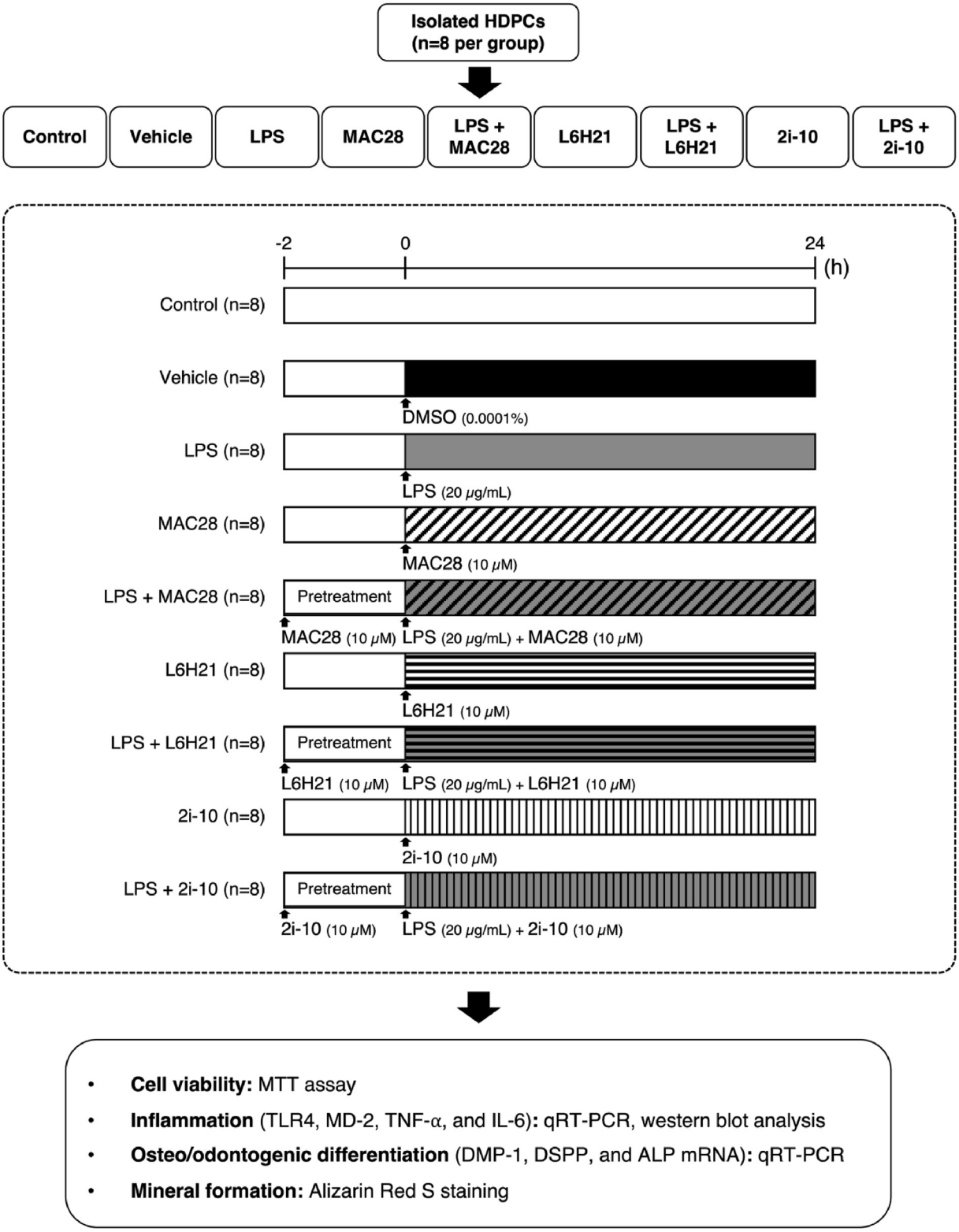

Figure 2 Flow chart of the experimental protocol. ALP, alkaline phosphatase; DMP-1, dentin matrix protein-1; DMSO, dimethyl sulfoxide; DSPP, dentin sialophosphoprotein; HDPCs, human dental pulp cells; IL-6, interleukin 6; LPS, lipopolysaccharides; MD-2, myeloid differentiation factor 2; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; qRT-PCR, quantitative real-time polymerase chain reaction; TLR4, toll-like receptor 4; TNF-α, tumor necrosis factor alpha.

Table 1 List of primer pairs used for qRT-PCR analysis.

mRNA	Primer sequences (5′ to 3′)
TLR4	Forward: CAA CAA AGG TGG GAA TGC TT
	Reverse: TGC CAT TGA AAG CAA CTC TG
MD-2	Forward: TTC CAC CCT GTT TTC TTC CA
	Reverse: AAT CGT CAT CAG ATC CTC GG
TNF-α	Forward: GCT GCA CTT TGG AGT GAT CG
	Reverse: CTT ACC TAC AAC ATG GGC TAC AG
IL-6	Forward: ATG AAC TCC TTC TCC ACA AGC GC
	Reverse: GAA GAG CCC TCA GGC TGG ACT G
DMP-1	Forward: CAC TCA AGA TTC AGG TGG CAG
	Reverse: TCT GAG ATG CGA GAC TTC CTA AA
DSPP	Forward: AAT GGG ACT AAG GAA GCT G
	Reverse: AAG AAG CAT CTC CTC GGC
ALP	Forward: GTT CAG CTC GTA CTG CAT GTC
	Reverse: ATC GCC TAC CAG CTC ATG CAT
GAPDH	Forward: ACC ACA GTC CAT GCC ATC AC
	Reverse: TCC ACC ACC CTG TTG CTG TA

ALP, alkaline phosphatase; DMP-1, dentin matrix protein-1; DSPP, dentin sialophosphoprotein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL-6, interleukin 6; MD-2, myeloid differentiation factor 2; qRT-PCR, quantitative real-time polymerase chain reaction; TLR4, toll-like receptor 4; TNF-α, tumor necrosis factor alpha.

ab9324), and anti-β-Actin (mouse; Santa Cruz Biotechnology, Dallas, TX, USA; SC-47778), in conjunction with secondary antibodies. The membranes were visualized and scanned with the ChemiDoc™ Touch Gel Imaging System (Bio-Rad). The ImageJ 1.52a (Wayne Rasband, Bethesda, MD, USA) analysis program was used to evaluate the immunoblotting images.

Determination of osteo/odontogenic differentiation by qRT-PCR

Following a 24-h treatment period, osteo/odontogenic differentiation induction was performed by differentiation-inducing media, as narrated by Weekate and colleagues.[13] Regular α-MEM was used as a control. All media were replenished every three days. After osteo/odontogenic differentiation for 21 days, HDPCs were subjected to qRT-PCR using a procedure previously described by Ongnok and colleagues.[15] The primer pairs used for dentin matrix protein-1 (DMP-1), dentin sialophosphoprotein (DSPP), and alkaline phosphatase (ALP) are listed in Table 1.

Determination of mineralization by Alizarin Red S staining

Following a mineralization induction for 21 days, cell fixation with 4% paraformaldehyde was performed. Then, HDPCs were soaked in Alizarin Red S solution (Sigma—Aldrich) for 5 min at room temperature. After rinsing and being left dry for one week, the stained cells were de-stained by 10% methylpyridinium chloride monohydrate (Sigma-Aldrich).[13] The microplate reader (BioTek) was set at 540 nm for absorbance measurement in order to quantify the amount of mineralization.

Statistical analysis

For all experiments, n = 8 per group was used for statistical analysis. In each experiment, the cells from each donor were triplicated. Each parameter was measured thrice and finally calculated the average value of each donor from these three measurements, which was used for data analysis. Data were displayed as mean ± standard error of the mean (SEM). One-way ANOVAs followed by post-hoc LSD analysis was carried out for comparison between groups. All statistical analysis was managed by GraphPad Prism 8.2.1 software for Mac (Dennis Radushev, San Diego, CA, USA). A 95% confidence level was used in this study to measure statistical significance ($P < 0.05$).

Results

LPS, MAC28, L6H21, and 2i-10 did not alter the cell viability of HDPCs

LPS (20 μg/mL) did not decrease the amount of viable HDPCs at 24 h when compared to the control group ($P > 0.05$) (Fig. 3A). In addition, treatment with either 0.0001% of DMSO (vehicle group) or 10 μM of MAC28, L6H21, or 2i-10 for 24 h had no effects on the cell viability of HDPCs, when compared with the control group ($P > 0.05$) (Fig. 3A). HDPCs-pretreated with either 10 μM of MAC28, L6H21, or 2i-10 for 2 h, followed by incubation with 20 μg/mL LPS for 24 h did not affect the viability of HDPCs ($P > 0.05$) (Fig. 3A).

LPS increased inflammatory cytokines through upregulation of TLR4-MD-2 signaling

LPS (20 μg/mL) was used to promote an inflammatory response in HDPCs for 24 h. LPS-treated HDPCs exhibited a significant upregulation of the mRNA of TLR4 and MD-2 and their protein expressions when compared with the control and the vehicle groups ($P < 0.05$) (Fig. 3B—E). No difference in TLR4 and MD-2 mRNA levels or their protein expressions were found among the control and the vehicle groups ($P > 0.05$) (Fig. 3B—E). Moreover, an increase in TNF-α and IL-6 mRNA and protein expression, relative to the control and the vehicle group ($P < 0.05$) (Fig. 3F—I), were noticed in HDPCs activated with LPS. The vehicle and control groups also showed the same levels of TNF-α and IL-6 mRNA and protein expression (Fig. 3F—I).

MAC28, L6H21, and 2i-10 alleviated the LPS-induced inflammatory cytokines in HDPCs through suppression of TLR4 and MD-2 expression

Pretreatment with either 10 μM of MAC28 or L6H21 or 2i-10 followed by incubation with LPS attenuated the LPS-induced inflammation by reducing the levels of TLR4, MD-

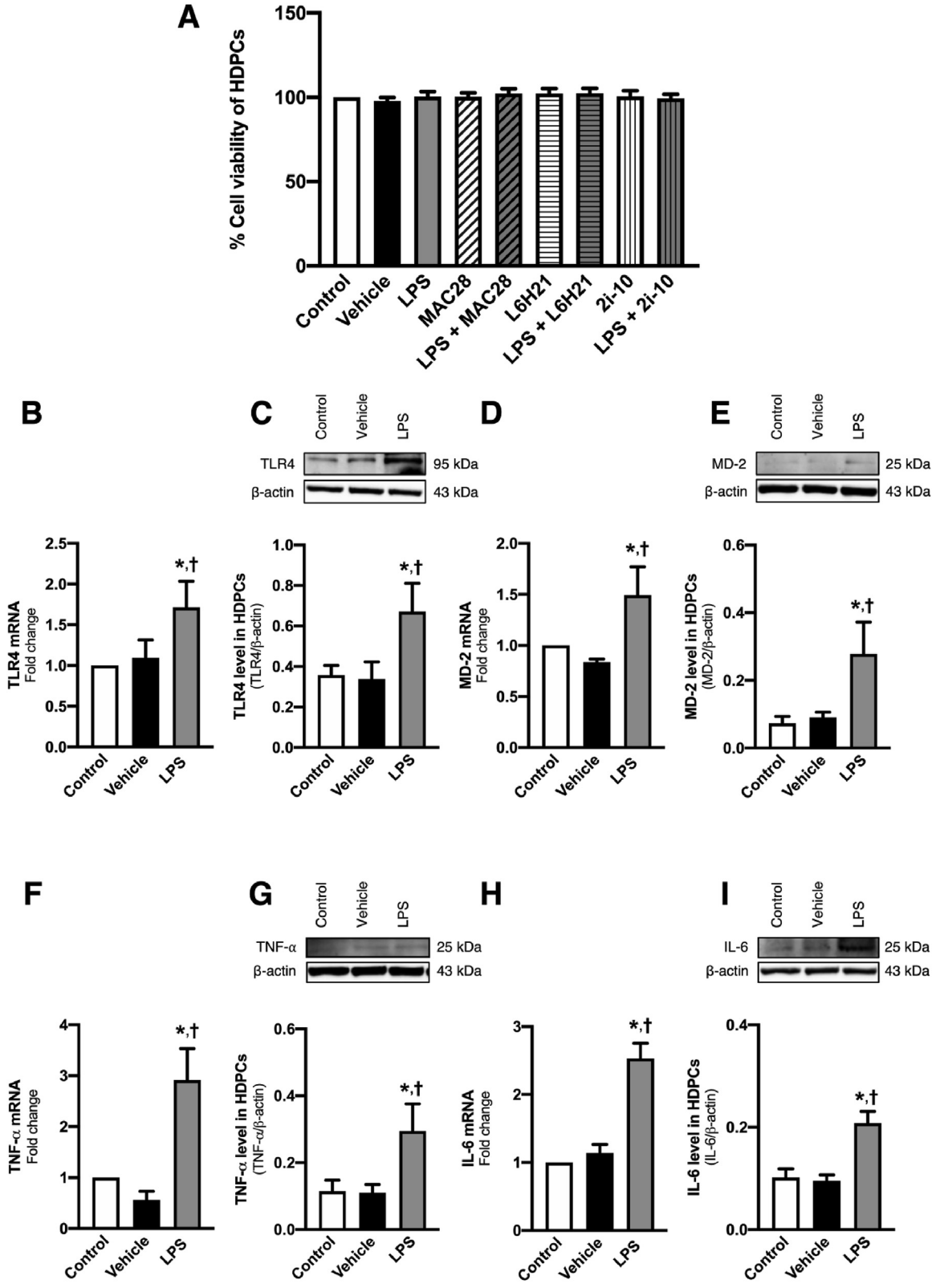

Figure 3 Cell viability and inflammatory profiles of LPS-treated HDPCs. (A) Cell viability (% control) of LPS-treated HDPCs with MAC28, L6H21, or 2i-10 pretreatment. (B) Expression of TLR4 mRNA. (C) Expression of TLR4 protein with representative images of immunoblotting. (D) Expression of MD-2 mRNA. (E) Expression of MD-2 protein with representative images of immunoblotting. (F) Expression of TNF-α mRNA. (G) Expression of TNF-α protein with representative images of immunoblotting. (H) Expression of IL-6 mRNA. (I) Expression of IL-6 protein with representative images of immunoblotting in LPS-treated HDPCs. Data are shown as mean ± SEM (n = 8/group). *$P < 0.05$ vs. control; †$P < 0.05$ vs. vehicle. HDPCs, human dental pulp cells; IL-6, interleukin 6; LPS, lipopolysaccharides; MD-2, myeloid differentiation factor 2; TLR4, toll-like receptor 4; TNF-α, tumor necrosis factor alpha.

2, TNF-α, and IL-6 mRNA and protein expression ($P < 0.05$) (Fig. 4A–H).

MAC28, L6H21, and 2i-10 improved osteo/odontogenic differentiation and mineralization in LPS-induced HDPCs

In this study, we successfully instigated osteo/odontogenic differentiation using differentiation media (Fig. 5A and B). Our results demonstrated that HDPCs exposed to LPS for 24 h before osteo/odontogenic differentiation significantly impaired mineralization when compared with the control/diff and the vehicle/diff groups ($P < 0.05$) (Fig. 5B). Interestingly, MAC28, L6H21, and 2i-10 could reverse the LPS-induced reduction of mineralization in HDPCs to the level observed in the vehicle/diff group ($P < 0.05$) (Fig. 5C and D).

Considering the mRNA representing osteo/odontogenic differentiation, the expression of the mRNA of DSPP, DMP-1, and ALP, which represented osteo/odontogenic differentiation, also decreased remarkably in the LPS/diff group when compared to those of the control/diff and the vehicle/diff groups ($P < 0.05$) (Fig. 5E–G). MAC28 and L6H21 were able to upregulate the levels of the mRNA of DSPP, DMP-1, and ALP mRNA to the same levels observed in the vehicle/diff group ($P > 0.05$) (Fig. 5H–J). However, 2i-10 restored only the level of DMP-1 mRNA ($P > 0.05$) (Fig. 5I), but not the expression levels of the mRNA of both DSPP and ALP, when compared to the vehicle/diff group ($P < 0.05$) (Fig. 5H and J).

Discussion

Important discoveries from this study were: 1) LPS triggered inflammation in HDPCs by upregulating MD-2, TLR4, and their associated inflammatory cytokines TNF-α and IL-6; 2) MAC28, L6H21, and 2i-10 inhibited inflammation equally by suppression of the TLR4-MD-2 signaling pathway in LPS-treated HDPCs; and 3) all three compounds could instigate recovery of mineralization and osteo/odontogenic differentiation in LPS-treated HDPCs.

LPS is one of the potent ligands for the activation of the TLR4-MD-2 signaling.[5] When LPS coheres with MD-2, this resulting complex initiates TLR4-MD-2 dimerization, which propagates intracellular signaling, resulting in the release of the inflammatory cytokines.[2] Studies have demonstrated that LPS could upregulate the TLR4 mRNA in various cells such as human dental pulp stem cells[6] and human aortic smooth muscle cells.[17] In addition, the over expression of the TLR4 on the cell membrane of human brain vascular pericytes, human mesenchymal stem cells, and mouse bone marrow-derived macrophages following LPS treatment were also observed.[18,19] Upon the LPS stimulation, the TLR4-myeloid differentiation factor 88 (MyD88) downstream signaling is activated which triggers the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and mitogen-activated protein kinase (MAPK) signaling pathways in cells. These processes initiate the translocation of transcriptional factors NF-κB and activator protein 1 (AP-1) into the nucleus, leading to the transcription of TLR4 gene.[20] MD-2 is essential in the upregulation of NF-κB through TLR4-MyD88 signaling following LPS stimulation. With the presence of MD-2, the NF-κB activation enhances remarkably in response to LPS treatment when compared to the MD-2 depletion condition.[3] Previous studies also reported that expression of MD-2 increased cell surface expression of TLR4.[21,22] In addition, Lin and colleagues showed that the upregulation of TLR4 mRNA could occur through the activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and MAPK signaling pathways following the LPS challenge in vascular smooth muscle cells.[23] Lin and colleagues also discovered that LPS could upregulate the RNA-binding protein human antigen R (HuR) which helped stabilizing intracellular TLR4 mRNA.[17] Apart from these novel findings, Tang and colleagues also found that the epidermal growth factor receptor (EGFR) was involved in the TLR4 upregulation following LPS stimulation in mouse macrophages since the phosphorylation of the EGFR was triggered after LPS treatment. Blocking the EGFR using EGFR inhibitor PD168393 both in *in vitro* and *in vivo* studies resulted in a decrease in TLR4 expression on the cell surface of mouse macrophages.[19] Interestingly, Tang and colleagues discovered that the EGFR play part in the TLR4 transportation from Golgi apparatus to the cell surface of macrophages.[19] Furthermore, Nagai and colleagues found that MD-2 was essential in promoting TLR4 on the cell surface since TLR4 was found to accumulate in Golgi apparatus when MD-2 was absence.[24] These findings were supported by Fujimoto and colleagues as they found that MD-2 reacted with TLR4 at the amino-terminal region for TLR4 transportation.[22] Taken together, these findings confirmed the role of MD-2, NF-κB, MAPK, and NADPH oxidase signaling pathways in promoting TLR4 mRNA following LPS stimulation.[20,21,23] The HuR also assist in stabilizing TLR4 mRNA[17] while EGFR and MD-2 promote transportation of TLR4 protein to the cell surface.[19,24] When the TLR4-MD-2 signaling is over-activated for a certain period, it triggers inflammation and associated pathologies including cell death.[2] In line with previous studies, we demonstrated that LPS induced an increase in mRNA levels and expressions of the proteins MD-2 and TLR4, and increased inflammatory cytokines, for example, TNF-α and IL-6, in the dental pulp cells.

When MD-2 in the TLR4-MD-2 signaling is inhibited by the MD-2 blockers MAC28 and L6H21, inflammation decreases.[1,10] In agreement with our study, Zhang and colleagues found that the curcumin analog MAC28 (10 μM) remarkably suppressed the expression of TNF-α, IL-6, IL-1β, and cyclooxygenase-2 (COX-2) mRNAs in the LPS-induced mouse primary peritoneal macrophages to a greater extent than the natural curcumin with similar concentration.[10] The efficacy of MAC28 was confirmed in LPS-induced acute lung injury rats in which the inflammation in lung tissues was reduced following the administration of MAC28 (20 mg/kg/day, P.O.).[10] Upon the compete binding of MAC28 to LPS at the hydrophobic pocket of the MD-2 at the Arg^{90} site (KD value = 59.5 μM), this prevents the TLR4-MD-2 signal transduction, resulting in the inflammatory suppression.[10] Similarly, previous studies examining the

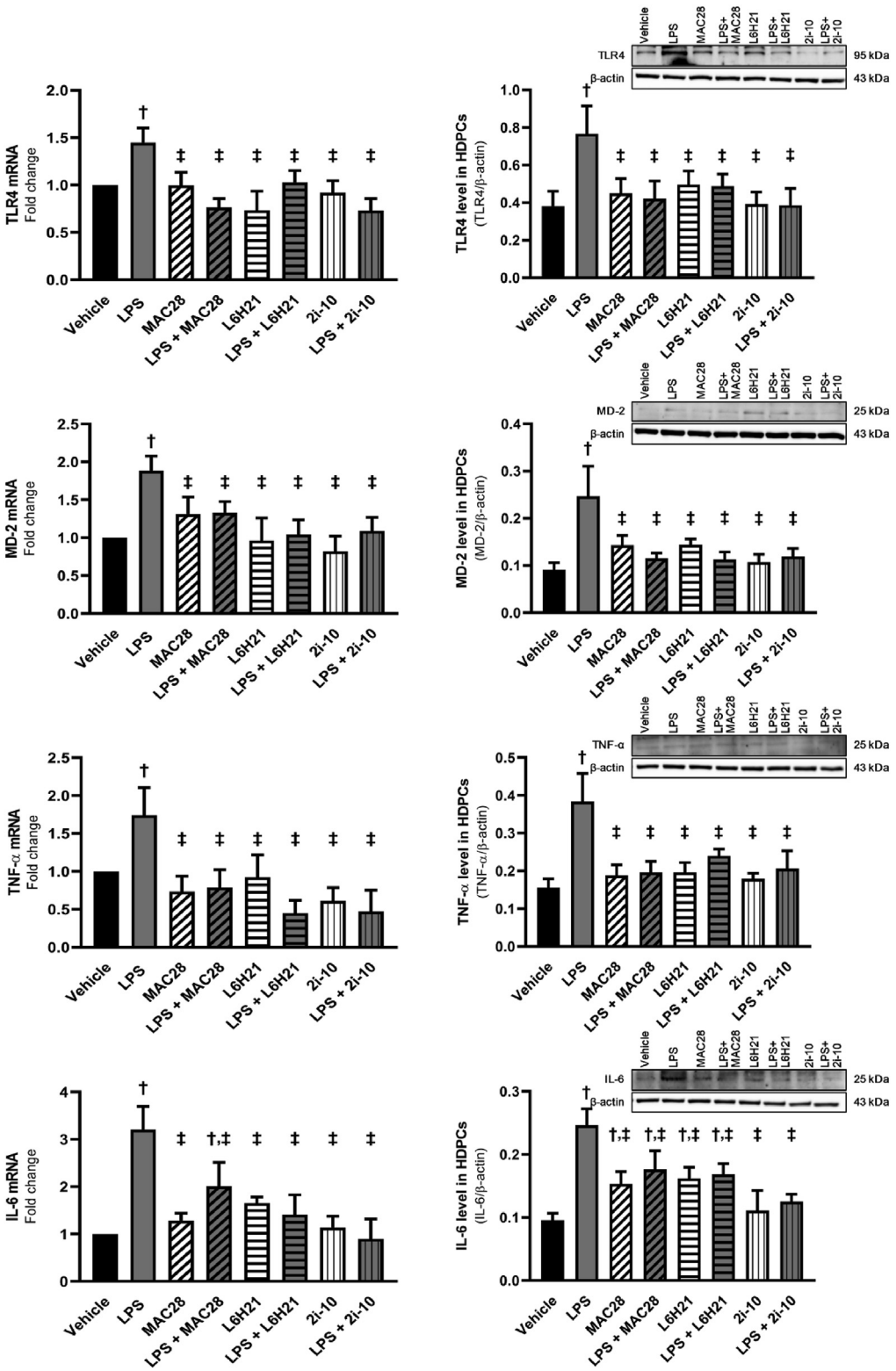

Figure 4 Inflammatory markers of LPS-treated HDPCs with MAC28, L6H21, or 2i-10 pretreatment. (A) Expression of TLR4 mRNA. (B) Expression of TLR4 protein with immunoblotting representation. (C) Expression of MD-2 mRNA. (D) Expression of MD-2 protein with immunoblotting representation. (E) Expression of TNF-α mRNA. (F) Expression of TNF-α protein with immunoblotting representation. (G) Expression of IL-6 mRNA. (H) Expression of IL-6 protein with immunoblotting representation in LPS-treated HDPCs. Data are shown as mean ± SEM (n = 4–6/group). $^{\dagger}P < 0.05$ vs. vehicle; $^{\ddagger}P < 0.05$ vs. LPS. HDPCs, human dental pulp cells; IL-6, interleukin 6; LPS, lipopolysaccharides; MD-2, myeloid differentiation factor 2; TLR4, toll-like receptor 4; TNF-α, tumor necrosis factor alpha.

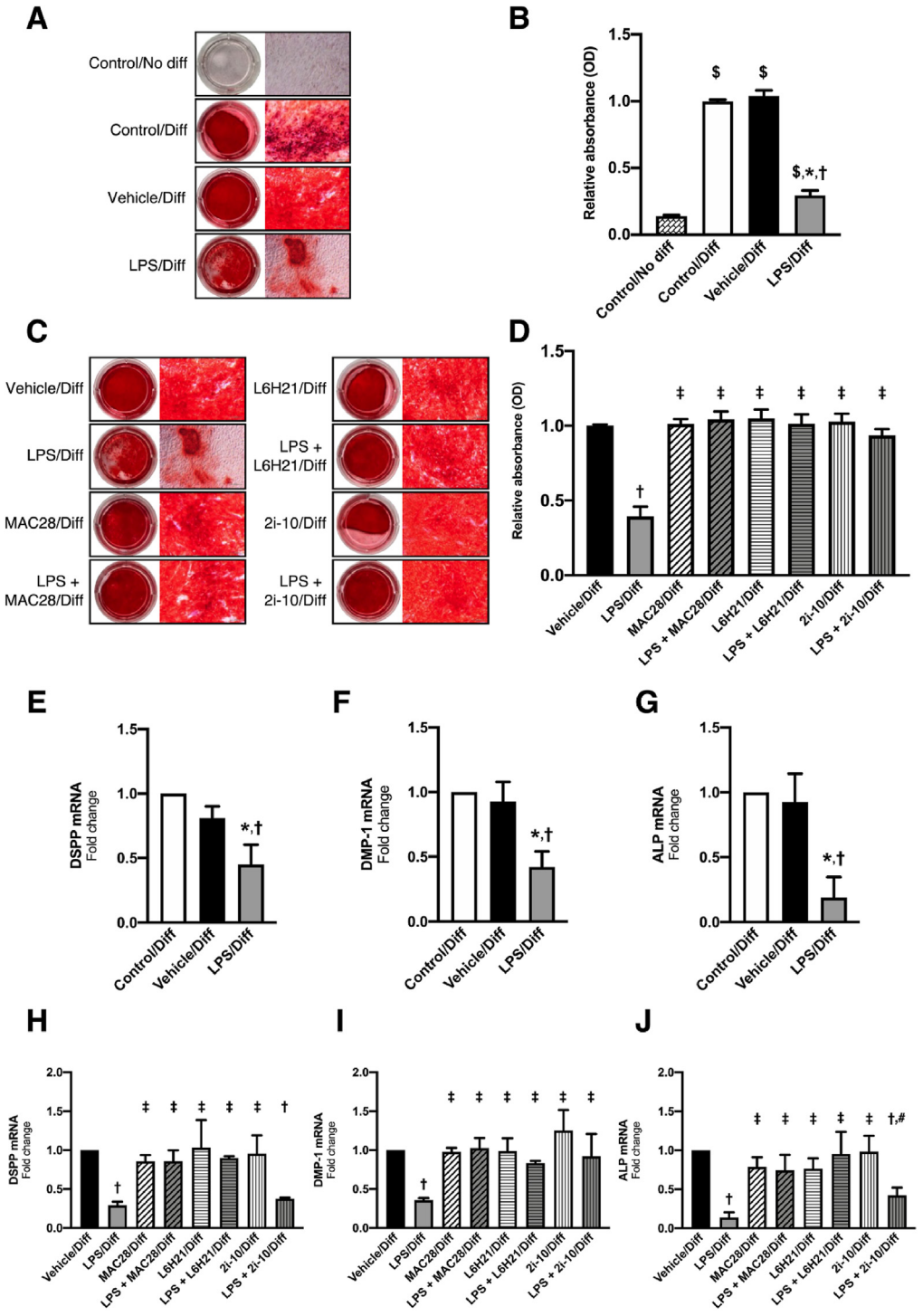

Figure 5 The evaluation of mineralization and osteo/odontogenic differentiation genes in LPS-treated HDPCs. (A) Representative images of Alizarin Red S staining of HDPCs in the control/no diff group in comparison with differentiated HDPCs in the control/diff, vehicle/diff, and LPS/diff groups. (B) Optical density values of mineral deposition in LPS-treated HDPCs in relation to the control/no diff, control/diff, and vehicle/diff groups. (C) Representative images of Alizarin Red S staining of LPS-treated HDPCs with MAC28, L6H21, or 2i-10 pretreatment. (D) Optical density values of mineral deposition in LPS-treated HDPCs with MAC28, L6H21, or 2i-10 pretreatment in relation to the vehicle and LPS groups. (E) Expression of DSPP mRNA in LPS-treated HDPCs. (F) Expression of DMP-1 mRNA in LPS-treated HDPCs. (G) Expression of ALP mRNA in LPS-treated HDPCs. (H) Expression of DSPP mRNA in LPS-treated HDPCs with MAC28, L6H21, or 2i-10 pretreatment. (I) Expression of DMP-1 mRNA in LPS-treated HDPCs with MAC28, L6H21, or 2i-10 pretreatment. (J) Expression of ALP mRNA in LPS-treated HDPCs with MAC28, L6H21, or 2i-10 pretreatment. Data are shown as mean ± SEM (n = 8/group). $^{\$}P < 0.05$ vs. control/no diff; $^{*}P < 0.05$ vs. control/diff; $^{\dagger}P < 0.05$ vs. vehicle/diff; $^{\ddagger}P < 0.05$ vs. LPS/diff; $^{\#}P < 0.05$ vs. LPS + L6H21/diff. ALP, alkaline phosphatase; Diff, differentiation; DMP-1, dentin matrix protein-1; DSPP, dentin sialophosphoprotein; HDPCs, human dental pulp cells; LPS, lipopolysaccharides; OD, optical density.

pharmacological effects of the chalcone derivative L6H21 showed that L6H21 (10 mg/kg, I.V.) could inhibit inflammation in heart and lung tissues by binding to the MD-2 at the Arg^{90} and the Tyr^{102} sites (KD value = 33.3 μM).[1] Although MAC 28 and L6H21 had been shown to minimize inflammation in LPS-treated macrophages,[1,10,25] no study has been done in dental pulp cells. Our study found that MAC28 and L6H21 could reverse the LPS-induced inflammation and restore the impaired osteo/odontogenic differentiation and mineral deposition in LPS-treated HDPCs.

In this study, the cinnamamide derivative 2i is also introduced as an MD-2 inhibitor.[11] The 2i exhibited its potential to disrupt the TLR4-MD-2 signaling in the LPS-treated macrophages by binding to the MD-2 at the Arg^{90} and the Tyr^{102} sites (KD value = 34 μM).[11] Furthermore, 2i pretreatment (10 mg/kg, I.V.) displayed its efficacy in preventing death in LPS-induced sepsis mice.[11] Recently, 2i-10, an analog of 2i, has been introduced. Our study found, for the first time, that 2i-10 reduced TLR4 and MD-2 expression while enhancing osteo/odontogenic differentiation and mineralization in LPS-treated HDPCs. However, only DMP-1 mRNA was increased by 2i-10 but the mRNA for DSPP and ALP mRNA were not. Secretory and mature odontoblasts secreted DSPP whereas DMP-1 was found in secretory and mature odontoblasts, osteoblasts, osteocytes, and cementoblasts.[26] As a dentin-like structure is preferable to a bone-like structure in regenerative dentistry, more research is needed to see if these three compounds can restore odontoblast-like cells and a dentin-like structure in inflamed dental pulp tissues or cells.

MAC28, L6H21, and 2i-10 showed similar efficacy in LPS-treated HDPCs as anti-inflammatory and osteo/odontogenic differentiation-promoting reagents. MD-2 inhibitors have already been established in the case of MAC28 and L6H21,[1,10,25] however, data for 2i-10 is not currently available. Before concluding that 2i-10 is an MD-2 inhibitor, more research should be done to confirm the molecular binding site of 2i-10 on MD-2.

Further studies involving the MD-2 inhibitors are still in need before any translational application can be made regarding the dose and administration of the MD-2 inhibitors under inflammation condition. More importantly, researchers should take into consideration that since MAC28, L6H21, and 2i-10 are hydrophobic, the drug preparations and delivery might be problematic. From previous studies and our study, the three MD-2 inhibitors were dissolved in less than or equals to 0.1% DMSO.[1,10,11] Although the DMSO at a very low concentration (≤0.1%) showed no adverse effects,[1,10,11] careful considerations on the adverse reactions should be taken closely in future studies. Furthermore, the investigation on the non-cytotoxic drug preparations and delivery system at pre-clinical and clinical levels of MD-2 inhibitors should be determined.

In conclusion, this is the first study to report the efficacy of MAC28, L6H21, and 2i-10 in the attenuation of inflammation through downregulation of TLR4 and MD-2 and the restoration of osteo/odontogenic differentiation and mineralization in LPS-treated HDPCs. These three compounds could be used as a possible treatment for dental pulpitis.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

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