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Aspirin enhances the osteogenic and anti-inflammatory effects of human mesenchymal stem cells on osteogenic BFP-1 peptide-decorated substrates†

Yan Li,[ab] Zuyuan Luo,[b] Xiao Xu,[a] Yongliang Li,[a] Siqi Zhang,[b] Ping Zhou,[a] Yi Sui,[a] Minjie Wu,[c] En Luo*[d] and Shicheng Wei *[ab]

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Several bone diseases, including arthritis, fracture and osteoporosis, have a pathophysiologically important inflammatory component. Sustained inflammation can result in delayed bone healing. Therefore, to promote bone repair, it is important to inhibit inflammatory bone erosion and suppress pro-inflammatory mediators. In this study, aspirin significantly enhanced immunomodulation and osteogenic differentiation in human mesenchymal stem cells (hMSCs). Additionally, an osteogenic BFP-1 peptide-decorated substrate (PS-PEP) enhanced osteogenic differentiation of aspirin-treated hMSCs compared to a pristine substrate. Alkaline phosphatase assay, quantitative real-time polymerase chain reaction, immunostaining and Alizarin Red S staining revealed that aspirin-treated hMSCs cultured on PS-PEP exhibited enhanced osteogenesis compared with untreated cells. Thus, we report here that the anti-inflammatory and osteogenic effects of aspirin promote the activity and osteogenesis of hMSCs. The combination of aspirin and an osteogenic BFP-1 peptide-decorated substrate suppresses the production of pro-inflammatory mediators and promotes osteogenic differentiation of hMSCs; therefore, this novel strategy has potential for application in cell therapy and bone tissue engineering.

1. Introduction

The incidence of bone defects associated with osteoarthritis, osteoporosis and tumours is increasing due to the aging of the population.[1] All organisms must protect themselves against exogenous pathogens and repair tissue damage resulting from infection or trauma.[2] However, the inflammatory response itself damages host tissue and causes organ dysfunction. For example, bone diseases are associated with sustained inflammation and delayed bone healing. Bone healing is promoted by the timely resolution of early inflammation. However, promotion or prolongation of inflammation impairs bone healing and causes bone destruction.[3] Additionally, over-production of pro-inflammatory mediators results in chronic, systemic inflammation, which suppresses bone healing and leads to joint destruction, such as in osteo- or rheumatoid arthritis.[4,5] Bone healing involves an inflammatory phase followed by bone formation and remodelling.[6] Inflammation is induced immediately after tissue damage due to fracture, surgical intervention or implantation. Osteoblasts secrete various pro-inflammatory mediators in an inflammatory environment, which either sustain the inflammation or contribute to bone repair.[7] However, bone biomaterials are typically developed by direct stimulation of osteogenic differentiation.[8] Therefore, research has focused on optimising the physicochemical and biomechanical properties of substrates to induce osteogenic differentiation, while neglecting the role of the immune response. Yang reported delayed bone healing in interleukin (IL)-6 knockout mice,[9] confirming the importance of eliminating the inflammatory response. The challenges in targeting inflammation in chronic inflammatory diseases are twofold: redundancy and compensation, which are critical for evolutionary survival.[10] Inflammation is orchestrated by several factors; targeting only one or a few of these may be insufficient. Moreover, inflammation involves multiple sensors and feedback pathways, so inhibition of a critical component of inflammation may simply trigger a compensatory pro-inflammatory response involving another pathway. Accordingly, a moderate,

[a] Central Laboratory, School and Hospital of Stomatology, Peking University, National Engineering Laboratory for Digital and Material Technology of Stomatology, Beijing 100081, China. E-mail: sc-wei@pku.edu.cn; Fax: +86 10 82195771; Tel: +86 10 82195771
[b] Laboratory of Biomaterials and Regenerative Medicine, Academy for Advanced Interdisciplinary Studies, Peking University, Beijing 100871, China
[c] 2nd Dental Center, Peking University School and Hospital of Stomatology, Beijing 100081, China
[d] State Key Laboratory of Oral Diseases, Department of Oral and Maxillofacial Surgery, West China School and Hospital of Stomatology, Sichuan University, Chengdu 610041, China. E-mail: luoen521125@sina.com

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controlled and rapidly resolved inflammatory reaction is essential for physiological bone healing.

Aspirin, a nonsteroidal anti-inflammatory drug (NSAID), has antipyretic, analgesic and inflammatory effects.[11,12] It inhibits all isoforms of COX by forming an irreversible covalent bond with the hydroxyl group of serine 530 (acetylation), blocking access to arachidonic acid.[11,12] Aspirin also reportedly reduces the risk of cancer and heart disease.[13–16] Aspirin has been reported to exert anti-postmenopausal osteoporosis effects in an ovariectomised rat model,[17–19] suggesting its potential clinical application for preventing bone loss. The frequent use of aspirin in the elderly population (who are susceptible to bone loss) and its effects on prostaglandin E2 (PGE2, a regulator of bone metabolism, can stimulate bone resorption by a cyclic AMP-dependent pathway) production by COX suggest that it could influence bone health.

Accordingly, we hypothesised that the cell state is important for osteogenic differentiation. In this study, aspirin was used to regulate the activity of human mesenchymal stem cells (hMSCs) at the initial stage of inflammation and stimulate their osteogenic differentiation later. Kim *et al.* demonstrated that the bone-forming peptide BFP-1 [GQGFSYPYKAVFSTQ] from bone morphogenetic protein-7 (BMP-7) had greater osteogenic activity than BMP-7 and induced osteogenesis in hMSCs.[20,21] Thus, we developed an osteogenic BFP-1 peptide-decorated substrate (PS-PEP) and assessed its effect on osteogenesis *in vitro* assisted by polydopamine (PDA) and carboxymethyl chitosan (CMC) technology. An intensively attached PDA film will be formed on the surfaces by simply immersing any solid substrate into dopamine solution under slightly alkaline conditions. However, the PDA film possesses intense binding ability with thiol, amine and imidazole groups,[22] which could affect the orientation and three-dimensional structure of biomolecules that contain these groups, and result in reduced or even abolished bioactivity. Synthetic surfaces that present a carboxyl group had been widely applied to conjugate peptides or polymers.[23,24] CMC, a derivative of chitosan, not only has good biocompatibility, biodegradability and bioactivity, but also contains both amine and carboxyl groups.[25,26] In order to better immobilize the peptide onto the PDA-decorated substrate, CMC as a bridge was grafted onto PDA coating through the abundant primary amines of CMC reacting with the oxidized catechol groups for tethering a large amount of peptides.[27] To our knowledge, this is the first report to describe that bone biomaterials (PS-PEP) in combination with aspirin-treated hMSCs can reduce inflammation and promote osteogenesis (Fig. 1). This strategy has potential for application in bone tissue engineering and cell therapy.

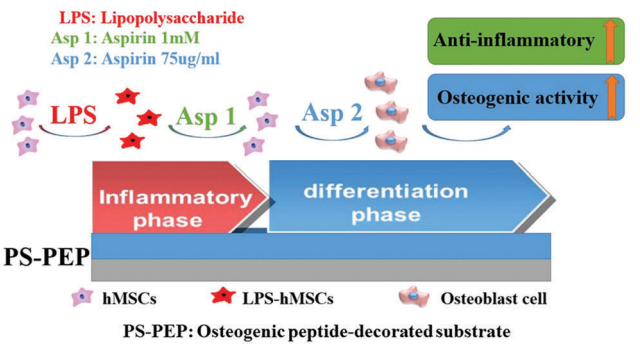

Fig. 1 Schematics of aspirin-administered hMSCs on osteogenic peptide-decorated substrates and anti-inflammatory and osteogenic activity assays.

2. Experimental

2.1. Materials

Aspirin and lipopolysaccharides (LPS) were purchased from Sigma-Aldrich Co. Tris(hydroxymethyl)-aminomethane (Tris–HCl) was provided by Sinopharm Chemical Reagent Co. Ltd (Beijing, China), and dopamine hydrochloride (DA) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Carboxymethyl chitosan (CMC), *N*-hydroxysuccinimide (NHS), *N*-(3-(dimethylamino)-propyl)-*N*′-ethylcarbodiimide hydrochloride (EDC) and 2-(*N*-morpho)ethanesulphonic acid (MES) were purchased from Aladdin (Shanghai, China). The BFP-1 peptide (Ac-KGGQGFSYPYKAVFSTQ) was synthesised using a batch-wise Fmoc-polyamide method by Chinapeptides Co. Ltd (Shanghai, China) and was of >98% purity as per the high-pressure liquid chromatography profile. All reagents were of analytical grade. All aqueous solutions were prepared in deionised (DI) water.

2.2. Preparation of osteogenic BFP-1 peptide-decorated substrates

Peptide decoration of substrates was performed as described previously.[28] Briefly, six-well PS cell culture plates (Corning Inc., Corning, NY, USA) were immersed in a dopamine solution (2 mg mL^{-1} in 10 mM Tris-HCl; pH 8.5) and gently shaken at 37 °C overnight. The surface of PDA-coated PS (PS-PDA) was ultrasonically cleaned with sterile distilled water for 5 min to remove PDA particles. PS-PDA was immersed in 5 mL of 3% (w/v) CMC solution with shaking for 16 h at 37 °C. The treated substrates were then washed with DI water to remove physically absorbed CMC, hereafter referred to as PS-PDA-CMC (PS-CMC). To immobilise the BFP-1 peptide on the PS-CMC surface, the samples were pretreated with 2 mM EDC and 5 mM NHS in 0.1 M MES buffer (pH 5.6) for 40 min at room temperature (RT). Next, peptide solutions (1 mM) were added to the carboxyl-rich PS-CMC surfaces and incubated at 4 °C overnight. The PS-PEP substrates were washed with DI water and dried under nitrogen before characterisation (Fig. S1, ESI†).

2.3. Characterisation of the BFP-1 peptide-decorated substrate

PS with and without PDA/CMC/PEP modifications was characterised by contact angle goniometry, X-ray photoelectron spectroscopy (XPS), and scanning electron microscopy (SEM). Surface hydrophilicity was measured using a contact angle goniometer (SL200B; Kono, USA) at ambient temperature and humidity. Six droplets of DI water were placed at random locations on each sample and the means and standard deviations were calculated. Chemical composition was analysed by XPS (AXIS Ultra; Kratos Analytical Ltd).

The binding energies were calibrated based on the C 1s hydrocarbon peak at 284.8 eV and a quantitative analysis was done using CasaXPS software. The surface morphology of the treated substrates was visualised using a field-emission scanning electron microscope (FE-SEM, JSM-6701F, Japan) at an acceleration voltage of 20 kV. Prior to SEM, all samples were coated with gold for 1 min.

2.4. Anti-inflammatory activity assay

Human mesenchymal stem cells (hMSCs; ScienCell Research Laboratories) were maintained in a normal growth medium comprising Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Life Technologies, Carlsbad, CA, USA), 10% foetal bovine serum (FBS; Gibco), 1% PS (Gibco) and 2 mM L-glutamine (Gibco) under standard humidified conditions (37 °C, 5% CO_2). The culture medium was changed every 2 days. Upon reaching 80–90% confluence, the cells were washed twice with PBS. A serum-free medium was then added and the cells were incubated overnight under standard humidified conditions. The medium was next replaced with fresh serum-free DMEM containing LPS (which are large molecules and can elicit strong immune responses in animals, 1 μg mL^{-1}), and 0.1, 0.5, 1, 5 or 10 mM aspirin was added. Following incubation for 24 h, the supernatant was decanted in accordance with the instructions of tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) ELISA kits. Replicate six specimens were assayed and the means and standard deviations were calculated.

2.5. Cell viability assay

hMSCs were seeded at passage five (3.0×10^4 cells per well) in triplicate in 12-well PS cell culture plates (Corning Inc.), and maintained in 1 mL of medium with aspirin (50, 75, 100, 150, 200, 400 or 800 μg mL^{-1}) or standard culture medium. Cells were digested with 0.25% trypsin–EDTA (Invitrogen), resuspended in 1 mL of PBS and counted 5 days after seeding. The effect of aspirin on hMSC proliferation was assessed by an assay using a Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan). At days 1, 4 and 7 of culture, the CCK-8 reagent (10%) in the culture medium was added to each well, followed by incubation for 2 h in the dark. Next, 100 μL of the supernatant from each well was transferred to a 96-well cell culture plate and the absorbance at 450 nm was determined using a microplate reader (Model 680; Bio-Rad, Canada). Replicate three specimens were assayed and the means and standard deviations were calculated.

2.6. Enzyme-linked immunosorbent assay (ELISA)

When cells reached 80–90% confluence, the medium was replaced with fresh serum-free DMEM containing LPS (1 μg mL^{-1}), and aspirin (75 μg mL^{-1}) was added. The supernatant was decanted after 12, 24 and 48 h, in accordance with the instructions of TNF-α, IFN-γ, osteoprotegerin (OPG) and receptor activator of nuclear factor-κB ligand (RANKL) ELISA kits. Replicate six specimens were assayed and the means and standard deviations were calculated.

2.7. Cytoskeleton visualisation

Cells were washed with PBS and fixed with 4% (w/v) paraformaldehyde for 20 min. Following washing with PBS, the cells were permeabilised with 0.1% (v/v) Triton X-100 (Sigma) for 5 min and then stained with 5 μg mL^{-1} FITC-phalloidin. After washing with PBS, the samples were incubated for 5 min at RT in 10 μg mL^{-1} DAPI. Signals were visualised using a CLSM (LSM510, Carl Zeiss).

2.8. *In vitro* osteogenic differentiation of hMSCs

2.8.1. Osteogenic differentiation. Osteoinductive medium was prepared by supplementation of ascorbic acid (50 μg mL^{-1}; Sigma), β-glycerophosphate (10 mM; Sigma), dexamethasone (100 nM; Sigma), streptomycin (100 μg mL^{-1}; Amresco, USA) and penicillin (100 U mL^{-1}; Amresco) to the normal growth medium.

2.8.2. Osteogenic differentiation of hMSCs. hMSCs were seeded on pristine (PS) and BFP-1 peptide-decorated substrates and the culture medium was changed every 2 days. When hMSCs reached 80–90% confluence, the culture medium was replaced by osteoinductive medium. Osteogenic differentiation on pristine and BFP-1 peptide-decorated substrates was assessed by determining the alkaline phosphatase activity and calcium deposition, which are early and late markers, respectively, of osteoblast differentiation. Replicate six specimens were assayed and the means and standard deviations were calculated.

2.8.3. LPS-mediated osteogenic differentiation. hMSCs were stimulated with LPS (1 μg mL^{-1}) and then transformed into LPS-hMSCs (LPS group). Cells not treated with LPS were used as a negative control (CT group). When hMSCs reached 80–90% confluence, the culture medium was replaced with osteoinductive medium, and osteogenesis was assessed by determining the alkaline phosphatase activity and calcium deposition. Replicate six specimens were assayed and the means and standard deviations were calculated.

2.8.4. Aspirin-induced osteogenic differentiation. When hMSCs reached 80–90% confluence, the culture medium was replaced by osteoinductive medium; hMSCs on pristine substrates not treated with aspirin were used as a negative control. The culture medium was changed every 2 days.

2.8.5. Alkaline phosphatase (ALP) activity. ALP staining was performed using a BCIP/NBT ALP colour development kit (Beyotime Biotechnology, Shanghai, China), following the manufacturer's instructions. The ALP activity of hMSCs was quantified using an assay kit (NJJC-BIO, Nanjing, China). Briefly, after 7 and 14 days of cultivation, hMSCs were lysed with Triton X-100 (1%) for 1 h at 4 °C. The cell lysates (30 μL) were mixed with ALP assay working solution and assayed following the manufacturer's instructions. For normalisation, the total protein concentration was measured using a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific). Replicate six specimens were assayed and the means and standard deviations were calculated.

2.8.6. Quantitative real-time polymerase chain reaction (qPCR). After 14 days of culture, total mRNA was isolated from hMSCs using the TRIzol reagent (Invitrogen) and converted into cDNA using a Revert Aid First-Strand cDNA Synthesis Kit (Thermo). A quantitative real-time polymerase chain reaction (qPCR) was carried out using SYBR Green (Roche, USA) on an ABI 7500 RT-PCR instrument (Applied Biosystems, USA) in triplicate, with β-actin as a housekeeping gene. The primers

used were: Col1a1-(forward): 5′-AGACACTGGTGCTAAGGGAGAG-3′, Col1a1-(reverse): 5′-GACCAGCAACACCATCTGCG-3′; OCN-(forward): 5′-CCTGAAAGCCGATGTGGT-3′, OCN-(reverse): 5′-AGGGCAGC GAGGTAGTGA-3′; ALP-(forward): 5′-CAACCCTGGGGAGGAGAC-3′, ALP-(reverse): 5′-GCATTGGTGTTGTACGTCTTG-3′; OPG-(forward): 5′-CTGGAACCCCAGAGCGAAAT-3′, OPG-(reverse): 5′-GCCTCC TCACACAGGGTAAC-3′; RANKL-(forward): 5′-GGTTGGGCCAA GATCTCCAA-3′, RANKL-(reverse): 5′-TCCGGATCCAGTAAGG AGGG-3′; and β-actin-(forward): 5′-CCCAGAGCAAGAGAGG-3′ β-actin-(reverse): 5′-GTCCAGACGCAGGATG-3′. The comparative CT ($2^{-\Delta\Delta CT}$) method was employed to evaluate fold differences in gene expression between groups.

2.8.7. Immunofluorescence. At day 21, hMSCs on BFP-1 peptide-decorated substrates were subjected to immunofluorescence analysis. They were fixed with 4% (v/v) paraformaldehyde for 20 min and permeabilised with 0.1% (v/v) Triton X-100 (Solarbio, Beijing, China) for 30 min at RT. Next, hMSCs were incubated in 3% bovine serum albumin/PBS at 37 °C for 2 h to block nonspecific binding. Then, the substrates were incubated with primary antibodies (rabbit anti-OCN IgG; [USA] at 1 : 50 dilution and rabbit anti-Col1a1 IgG [CST] at 1 : 200 dilution) at 4 °C overnight. Cells were washed twice with PBS and then incubated with secondary antibodies (goat anti-rabbit 488 IgG and goat anti-rabbit 647 IgG [Invitrogen, USA]) at 1 : 500 dilution for 1 h in the dark. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI, 10 μg mL^{-1}; Sigma-Aldrich) for 5 min at RT. Cells were visualised immediately by CLSM (LSM5; Carl Zeiss, Jena, Germany).

2.8.8. Alizarin Red S (ARS) staining. Calcium mineralisation was evaluated at day 21 by staining with ARS (Sigma) following the manufacturer's instructions. Briefly, the cells were fixed with 4% paraformaldehyde for 30 min and washed with DI water. Next, the samples were immersed in ARS solution (2%, pH 4.2; Sigma) for 10 min at RT. After washing with DI water, the samples were captured using a scanner. To quantify calcium mineralisation, stained samples were incubated with hexadecylpyridinium chloride (1% w/v; Sigma-Aldrich) overnight with shaking to dissolve calcium, and the absorbance of the eluate at 550 nm was assessed using a microplate reader. Replicate six specimens were assayed and the means and standard deviations were calculated.

2.8.9. Induction of osteogenic differentiation of hMSCs on BFP-1 peptide-decorated substrates by aspirin. The adhesion, growth and differentiation of hMSCs cultured on BFP-1 peptide-decorated substrates *in vitro* were investigated. Between the growth and differentiation phases, LPS (1 μg mL^{-1}) was added to mimic the inflammatory reaction. Aspirin (1 mM) was used to inhibit the inflammatory reaction and adjust the growth state of inflammatory cells. Next, aspirin (75 μg mL^{-1}) was added and the cells were incubated under standard humidified conditions for 21 days. To evaluate the osteogenic activity of hMSCs, the ALP activity was assessed using an ALP assay reagent kit and enzyme-histochemical staining at days 7 and 14 using a BCIP/NBT ALP colour development kit following the manufacturer's instructions. Additionally, calcium mineralisation at day 21 was evaluated by ARS staining. Normal hMSCs on BFP-1 peptide-decorated substrates not treated with aspirin were used as a negative control. Replicate six specimens were assayed and the means and standard deviations were calculated.

2.9. Statistical analysis

Data are expressed as means ± standard deviations. One-way analysis of variance (ANOVA) and Tukey's *post hoc* test were used to determine the significance of differences among the groups, and p values <0.05 were considered indicative of statistical significance.

3. Results and discussion

3.1. Characterisation and functional assessment of BFP-1 peptide-decorated substrates

The chemical composition and morphology of the BFP-1 peptide-decorated substrates were characterised by contact angle goniometry, XPS and SEM. The water contact angle of a surface is indicative of its hydrophobicity. Pristine PS had a contact angle of ~60° (relatively hydrophobic), which decreased to ~32° after coating with PDA and to ~21° after CMC attachment (Fig. S2a, ESI†). This was likely due to the hydrophilic groups (–COOH, –OH and $–NH_2$) of the grafted polymers. Peptide immobilisation onto the CMC-modified surface resulted in a decrease of the contact angle to ~17°, likely due to the superhydrophilicity of the peptide.

Next, the XPS spectra of the samples were evaluated. Carbon and oxygen predominated in pristine PS, while a small amount of nitrogen was present due to contamination (Fig. S2b, ESI†). Successful anchoring of PDA was indicated by an increase in the N 1s and O 1s contents. These changes were amplified following the grafting of CMC onto the PDA-based substrate, indicating immobilisation of CMC. In the wide-scan spectrum, the appearance of a sodium signal (due to PBS) and an increase in the nitrogen peak (N 1s, 14.65%) on the surface of PS-PEP indicated successful attachment of the peptide. These results suggest successful conjugation of the peptide to the modified PS surfaces.

The surface roughness and morphology of the PS substrate were altered by PDA coating or PDA-mediated CMC and peptide immobilisation. SEM images showed that bare PS had a smoother surface morphology than modified PS (Fig. S2c, ESI†). PDA particles were observed on the surface of PS-PDA. Because CMC forms a surface macromolecule membrane, wrinkles and PDA particles were present on the PS-CMC surface. The addition of an osteogenic BFP-1 peptide slightly increased the surface morphology/roughness of PS-CMC substrates. Compared to pristine PS, PS-PEP enhanced osteogenic differentiation of hMSCs at day 21 (Fig. S3a and b, ESI†). Covalently grafted molecules do not easily leach out from surfaces; covalent chemical conjugation is preferred over physical adsorption in tissue engineering.[29] Therefore, PS-PEP has potential for application in surface engineering of bone-mimicking materials.

3.2. Suppression of osteogenic differentiation by LPS

LPS elicit strong immune responses in animals.[30,31] To establish a model of inflammation, hMSCs were stimulated with LPS (1 μg mL^{-1}) for 24 h. LPS inhibited osteogenic differentiation,

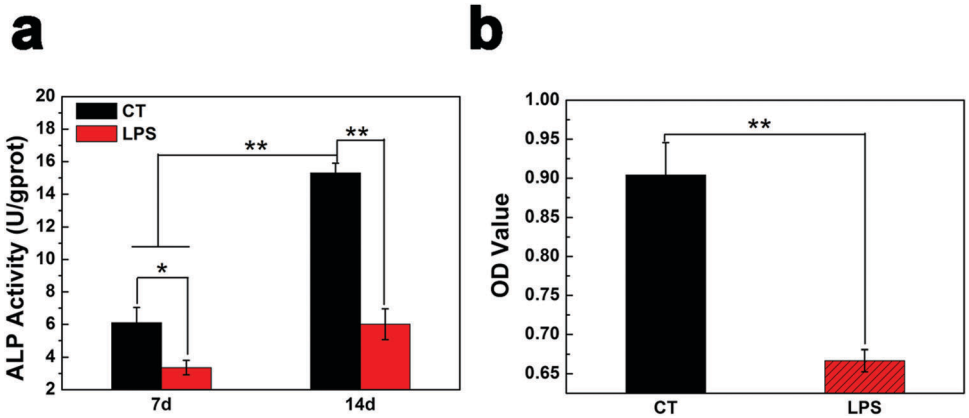

Fig. 2 Osteogenic differentiation in hMSCs cultured with or without LPS. (a) ALP expression at days 7 and 14. (b) Calcium deposition at day 21. CT, control; LPS, cells stimulated with LPS. (*) $p < 0.05$ and (**) $p < 0.01$.

suggesting that inflammatory cells exhibited significantly lower osteogenic differentiation potential than normal cells ($p < 0.01$) (Fig. 2a and b). Therefore, LPS treatment of hMSCs mimics an inflammatory environment, and the cell status affects osteogenic differentiation. Thus, cells must be in an appropriate state prior to the induction of osteogenesis.

3.3. Anti-inflammatory activity and cytocompatibility of aspirin

MSC-mediated bone formation is negatively correlated with the concentrations of TNF-α and IFN-γ.[32] TNF-α, a pro-inflammatory cytokine released by macrophages, is involved in periodontitis-mediated bone loss.[33] An elevated TNF-α level is associated with the severity of periodontal disease and the immune response.[34–36] Nuclear factor-κB (NF-κB), a *rel* family transcription factor found in multiple cell types,[37] is important in the pathogenesis of osteolysis due to inflammatory diseases such as rheumatoid arthritis, Paget's disease of bone (PDB), low-grade systemic inflammation, and some bacterial infections.[38] To maintain bone homeostasis, cytokines such as TNF-α and RANKL differentially regulate the classical and alternative NF-κB signalling pathways in

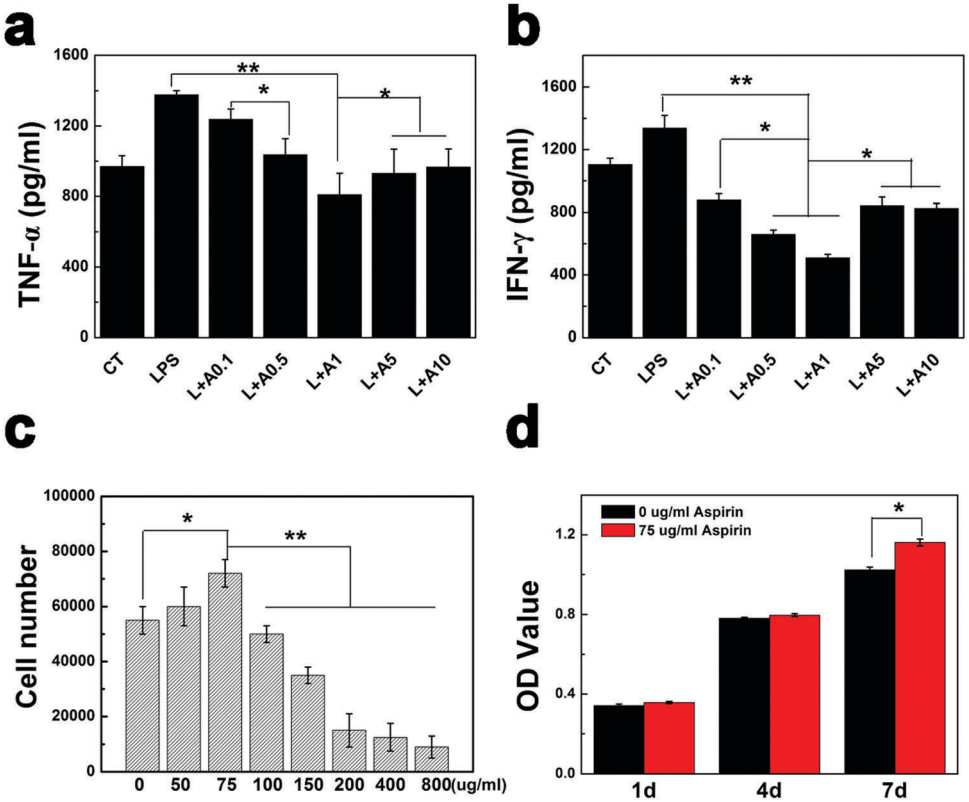

Fig. 3 Effect of aspirin (0.1, 0.5, 1, 5 and 10 mM) on pro-inflammatory cytokine expression and proliferation of hMSCs. (a) TNF-α. (b) IFN-γ. (c) Aspirin (75 μg mL^{-1}) enhanced hMSC proliferation. (d) Results are representative of at least three independent experiments. (*) $p < 0.05$ and (**) $p < 0.01$.

osteoclasts. TNF-α activates nuclear factor NF-κB, resulting in the upregulation of genes that regulate inflammation, proliferation and apoptosis.[39]

Aspirin and its analogues are among the most widely used drugs worldwide.[40] Aspirin inhibits the activation of NF-κB by stabilising inhibitor κB (IκB).[41] This led us to investigate the effect of aspirin (0.1, 0.5, 1, 5 and 10 mM) on NF-κB-regulated TNF-α expression. Aspirin (1 mM) treatment of hMSCs significantly decreased the TNF-α and IFN-γ concentrations compared with those in the LPS group (Fig. 3a and b). Additionally, hMSCs were co-cultured with 50, 75, 100, 150, 200, 400, or 800 μg mL^{-1} aspirin or standard culture medium for 5 days. Cells were then digested with 0.25% trypsin–EDTA, resuspended in 1 mL of PBS and counted. Aspirin at 75 μg mL^{-1} enhanced hMSC proliferation; however, higher concentrations (200, 400 and 800 μg mL^{-1}) inhibited such proliferation (Fig. 3c). CCK-8 assay confirmed that 75 μg mL^{-1} aspirin enhanced hMSC proliferation (Fig. 3d).

3.4. Effect of aspirin on the production of pro-inflammatory and osteogenic cytokines

The effect of aspirin (75 μg mL^{-1}) on TNF-α and IFN-γ production by LPS-stimulated hMSCs after culture for 12, 24 and 48 h was determined by ELISA. TNF-α is produced by activated macrophages and its concentration is increased during the acute phase of the immune response,[42,43] and IFN-γ downregulates the runt-related transcription factor 2 (Runx-2) pathway and enhances TNF-α signalling by stem cells.[26] Compared with those in the LPS group, TNF-α and IFN-γ concentrations were decreased by the local administration of aspirin (Fig. 4a and b); however, aspirin treatment significantly reduced the concentration of IFN-γ (Fig. 4b) ($p < 0.05$), but not that of TNF-α (Fig. 4a). The bone-protective effect of aspirin was likely due to the suppression of stimuli for osteoclast formation, as indicated by a reduction in RANKL mRNA and protein levels.[44] In contrast, aspirin increased the OPG mRNA and protein levels in bone. Rats treated with the combined regimen benefited from both effects, and exhibited a

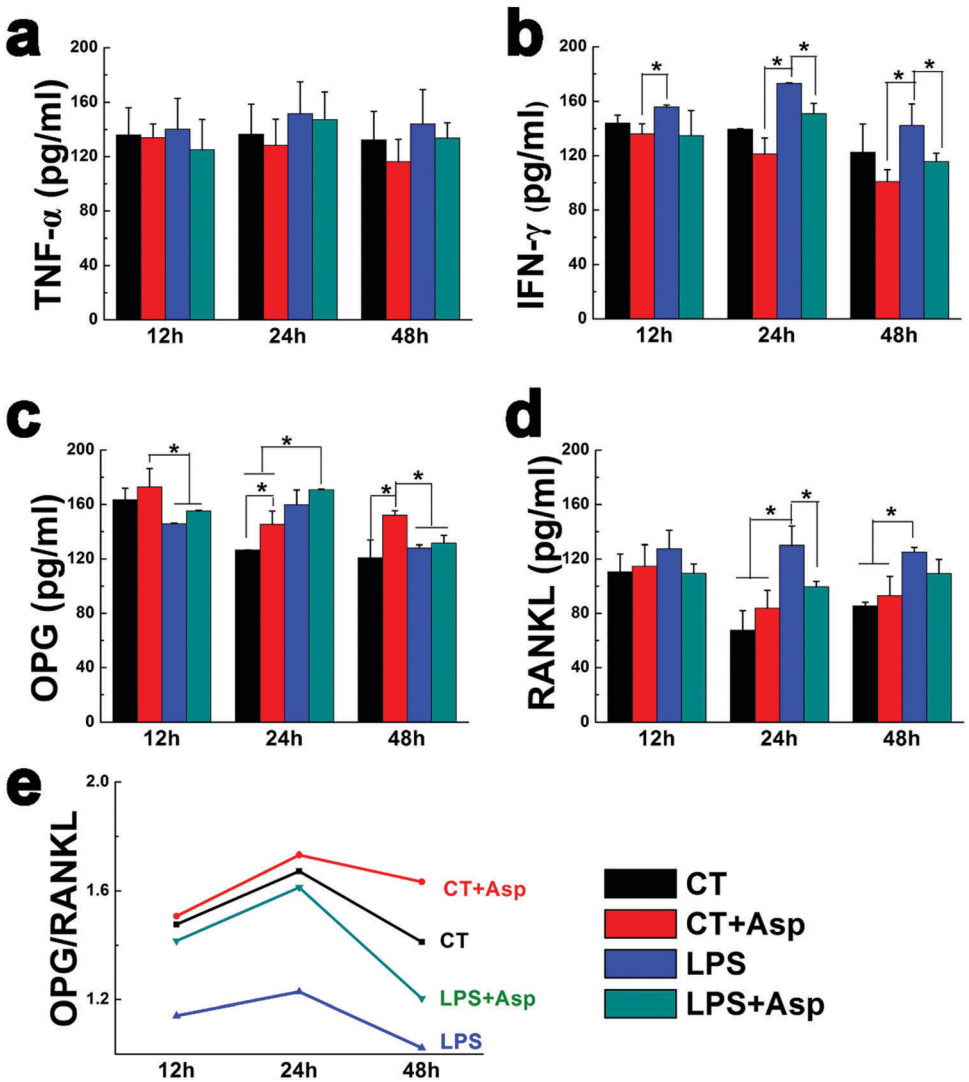

Fig. 4 Effect of aspirin (75 μg mL^{-1}) on the expression of TNF-α, IFN-γ, OPG and RANKL. CT, control; LPS, cells stimulated with LPS. Results are mean ± standard deviations (SD). (*) $p < 0.05$.

higher OPG/RANKL ratio than rats treated with the two agents individually.[38] To evaluate the osteogenic effects of aspirin (75 μg mL^{-1}), we investigated OPG and RANKL levels by ELISA. Compared with those in the control, aspirin treatment significantly increased the concentration of OPG (Fig. 4c) and decreased that of RANKL (Fig. 4d). The OPG/RANKL ratio was highest at 24 h (Fig. 4e). Therefore, treatment with aspirin for 24 h had a marked effect on osteogenic differentiation in hMSCs.

3.5. Morphology of adherent hMSCs

To confirm the biocompatibility of aspirin and PS-PEP, the morphology of adherent hMSCs was examined at 24 h (Fig. S4, ESI†). hMSCs cultured on PS not treated with aspirin were used as a control. Compared with the control group, cells on PS-PEP + Asp exhibited an elongated morphology with good adherence, spreading and mature F-actin intracellular stress fibres, suggesting that the BFP-1 peptide-decorated substrate and aspirin have good *in vitro* cytocompatibility, which enhances cell proliferation, spreading and morphology.

3.6. Alkaline phosphatase (ALP) and matrix mineralisation

Upregulation of ALP activity is a pivotal event in early osteogenesis.[1] Moreover, ALP is an important indicator of differentiating osteoblasts and osteogenesis at 7–14 days.[45,46] As shown in Fig. 5a, enzyme-histochemistry staining was conducted to visualize ALP distribution, and aspirin-treated hMSCs cultured on the BFP-1 peptide-decorated substrate showed greater crystallisation. Meanwhile, Fig. 5b shows that the PS-PEP + Asp group exhibited a significantly higher ALP activity compared with the other groups ($p < 0.01$), indicating that aspirin administration and PS-PEP are able to induce an up-regulation of ALP, correlated with the first check-point for osteogenic differentiation.

Calcium deposition at late stages of differentiation is an indication of osteogenesis by hMSCs.[47] At day 21, cells aggregated and formed bone-like structures that stained with ARS (Fig. 5c). Similar to the case for ALP activity, the BFP-1 peptide-decorated substrates harboured a larger number of calcium nodules, suggesting enhanced osteogenesis. Moreover, the PS-PEP + Asp group exhibited more intense red staining than the control group. Conjugation of peptides on the PS surface resulted in a significant increase in the amount of mineralised matrix. Aspirin-treated hMSCs cultured on BFP-1 peptide-decorated substrates showed the largest number of calcium nodules (Fig. 5d). This suggests that peptides are involved in the osteogenic differentiation of hMSCs.

3.7. Quantitative real-time PCR (qPCR) and immunofluorescence analyses

Initialisation and completion of cell differentiation are accompanied by regulation of the expression of multiple genes.

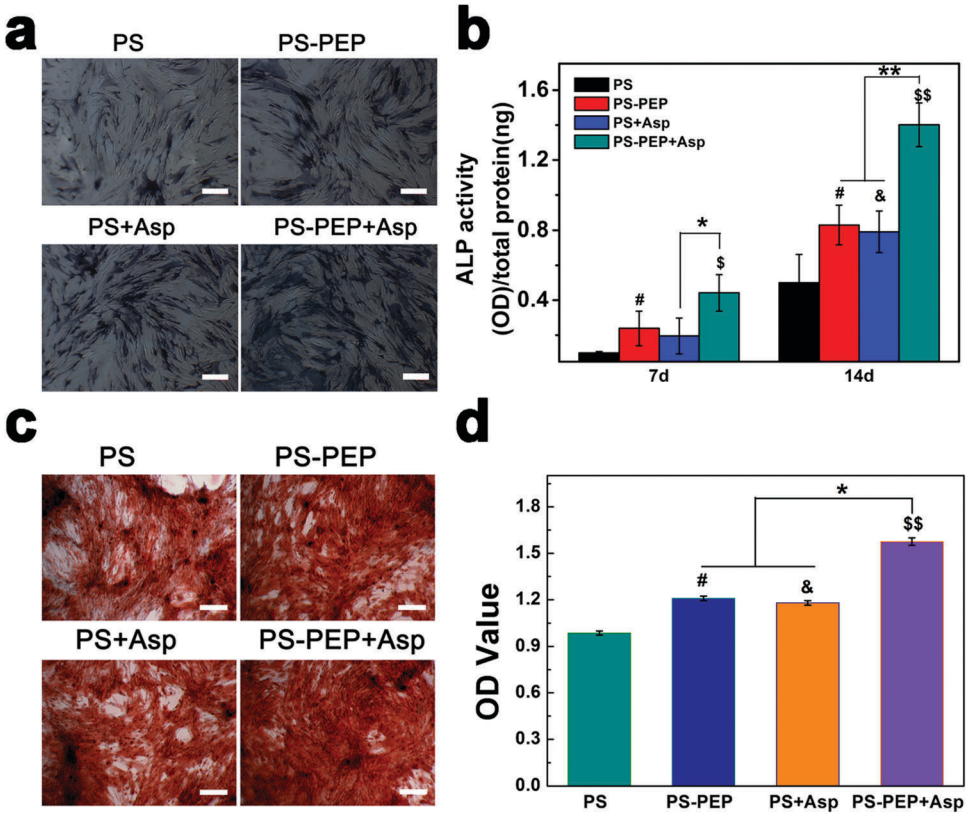

Fig. 5 Effect of aspirin (75 μg mL^{-1}) on ALP expression and calcium deposition by hMSCs on different substrates. (a) Representative image of ALP staining. (b) ALP activity at days 7 and 14. (c) ARS staining. (d) Calcium deposition at day 21. Compared to the PS group, #, & and $, significant the differences of PS-PEP, PS + Asp and PS-PEP + Asp groups, respectively (#, &, $ and * $p < 0.05$; $$ and **, $p < 0.01$). Scale bars indicate 200 μm.

Therefore, we evaluated osteocalcin (OCN), collagen type I alpha 1 (Col1a1), ALP, OPG and RANKL expression in hMSCs on BFP-1 peptide-decorated substrates at day 14. OCN and Col1a1 are markers of the mineralisation and production of organic bone matrix, respectively.[48] The fold change in OCN expression in hMSCs on BFP-1 peptide-decorated substrates was greater than that in the control group (Fig. 6a). Col1a1 and ALP expression was correlated well with that of OCN in the PS, PS-PEP, PS + Asp and PS-PEP + Asp groups (Fig. 6b and c). OPG and RANKL expression was also analysed. Bone formation is a complex process dependent on a balance between osteoblast formation and osteoclast resorption.[49] The crosstalk between osteoblasts and osteocytes is mediated by various intercellular signalling molecules. For example, osteoblasts secrete a receptor activator of the nuclear factor kappa-B (RANK) ligand (RANKL), which binds with RANK on preosteoclasts and stimulates their differentiation into osteoclasts. However, osteoblasts also release OPG as a decoy receptor to bind RANKL, which inhibits the differentiation of osteoclasts. In this study, OPG expression was found to correlate well with that of OCN, Col1a1 and ALP in the four groups. On the contrary, RANKL expression was not the same; even more, the expression of RANKL in the PS-PEP + Asp group was the lowest among the four groups (Fig. 6d and e). These results suggest that aspirin enhances the expression of OCN, Col1a1, ALP and facilitates the degradation of phospho-beta-catenin, thereby increasing Wnt signalling, which is essential for osteoblast formation.[50] Aspirin also prevents osteoclast formation by inhibiting the NF-κB pathway and enhances osteoblast formation by preventing apoptosis of its progenitor stem cells and stimulating preosteoblast differentiation. Therefore, local administration of aspirin could increase OPG expression and decrease RANKL expression.

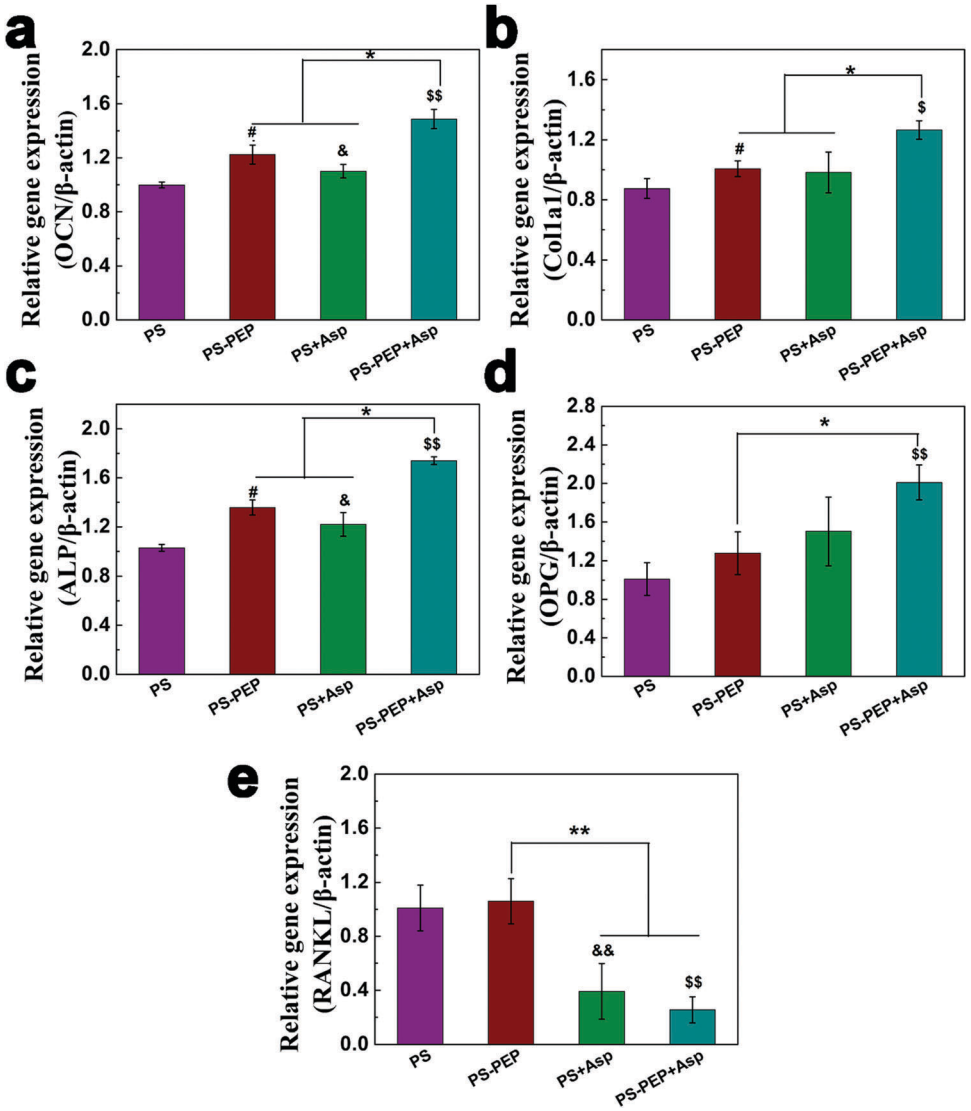

Fig. 6 Effect of aspirin (75 μg mL^{-1}) on the expression of osteogenic markers by hMSCs on different substrates. (a–e) RT-qPCR analysis of OCN, Col1a1, ALP, OPG and RANKL expression. Compared to the PS group, #, & and $, significant differences of PS-PEP, PS + Asp and PS-PEP + Asp groups, respectively (#, &, $ and * $p < 0.05$; $$, ** and &&, $p < 0.01$).

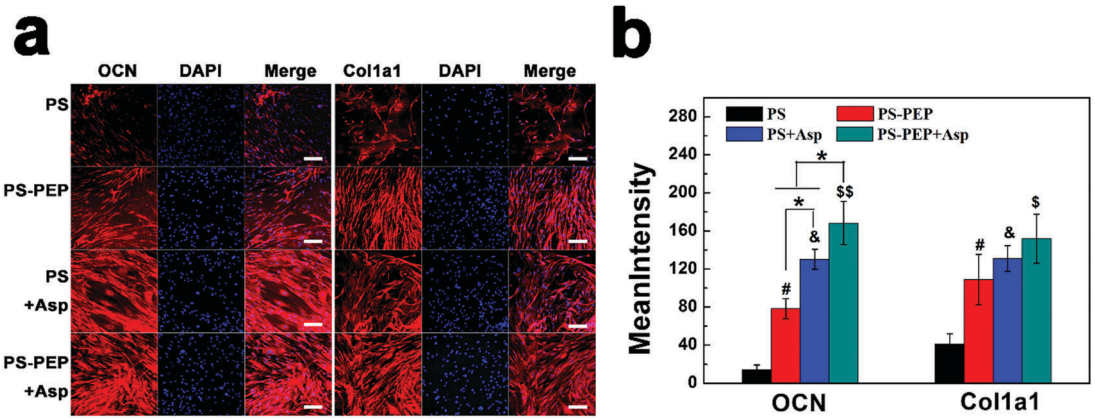

Fig. 7 Representative immunofluorescence images and signal mean intensity of OCN and Col1a1 at day 21. (a) OCN and Col1a1 are red, and nuclei are stained blue by DAPI. (b) Signal mean intensity quantified from OCN and Col1a1 images. Compared to the PS group, #, & and $, significant differences of PS-PEP, PS + Asp and PS-PEP + Asp groups, respectively (#, &, $ and * $p < 0.05$; $$, $p < 0.01$). PS, pristine; PS-PEP, substrates with osteogenic functions; Asp, aspirin 75 μg mL^{-1}. Scale bars indicate 200 μm.

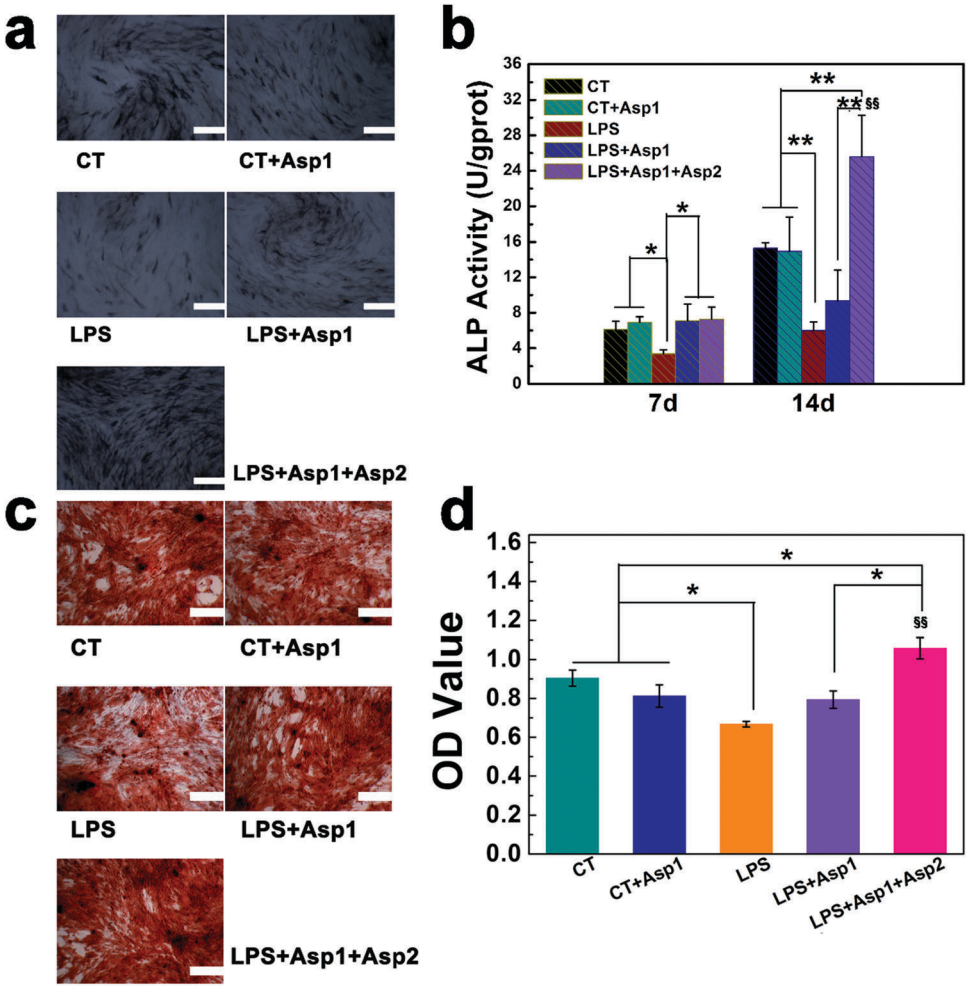

Fig. 8 Effect of aspirin on osteogenic differentiation of hMSCs on peptide-decorated substrates. (a) Representative image of ALP staining. (b) Determination of ALP activity at days 7 and 14. (c) ARS staining. (d) Determination of calcium deposition at day 21. CT, normal cells; LPS, cells stimulated with LPS (1 μg mL^{-1}); Asp1, 1 mM aspirin; Asp2, 75 μg mL^{-1} aspirin. § Significant differences between LPS and LPS + Asp1 + Asp2 groups, (§ and * $p < 0.05$; §§ and **, $p < 0.01$). Scale bars indicate 200 μm.

To corroborate the qPCR results, immunofluorescence analysis of OCN and Col1a1 was performed at day 21. The OCN and Col1a1 protein levels in the PS-PEP + Asp group were higher than those in the control group (Fig. 7a), in agreement with the qPCR findings. As shown in Fig. 7b, the signal mean intensity quantified from OCN and Col1a1 stained images suggested the same conclusion; moreover, the signal mean intensity from OCN shows significant differences among the groups of PS-PEP, PS + Asp and PS-PEP + Asp, but not that of Col1a1. Additionally, OCN and Col1a1 (red) staining in PS-PEP + Asp was the greatest among all groups, likely due to increased cell proliferation and ALP activity in the presence of a peptide and aspirin.

3.8. Effect of aspirin on osteogenesis on BFP-1 peptide-decorated substrates

The above results suggest that aspirin (1 mM) ameliorates inflammation and modulates the growth of inflammatory cells. Additionally, treatment of hMSCs with aspirin (75 μg mL^{-1}), as well as culture on BFP-1 peptide-decorated substrates, induced osteogenesis. Therefore, the differentiation of aspirin-treated hMSCs on BFP-1 peptide-decorated substrates was investigated *in vitro* by assessing ALP activity and calcium deposition. hMSCs cultured on BFP-1 peptide-decorated substrates and treated with LPS followed successively by 1 mM and 75 μg mL^{-1} aspirin exhibited greater crystallisation at day 14 (Fig. 8a). ALP activity was significantly increased in cells co-cultured with 1 mM and 75 μg mL^{-1} aspirin successively compared with that in the other groups ($p < 0.01$) (Fig. 8b). This is in accordance with the finding that the local administration of aspirin ameliorates inflammation at disease sites and promotes hMSC-induced regeneration by suppressing TNF-α and IFN-γ production.[51] The results above suggest that aspirin-treated hMSCs cultured on BFP-1 peptide-decorated substrates exhibit enhanced osteogenesis.

Next, we analysed mineralisation by ARS staining. Similar to ALP activity, the LPS group exhibited the lowest intensity of ARS staining at day 21 (Fig. 8c and d). Aspirin treatment resulted in a significant increase in the amount of mineralised matrix. hMSCs treated with 1 mM and 75 μg mL^{-1} aspirin on BFP-1 peptide-decorated substrates showed the highest number of calcium nodules. Taken together, these findings confirm that aspirin-treated hMSCs on BFP-1 peptide-decorated substrates stimulate osteogenic differentiation by enhancing mineralisation. Therefore, the combination of aspirin and an osteogenic BFP-1 peptide-decorated substrate has great potential for application in bone tissue engineering.

4. Conclusions

In summary, we reported here a novel strategy to promote osteogenesis by hMSCs, mediated by the anti-inflammatory and osteogenic differentiation effects of aspirin. Therapeutic doses of aspirin exert two effects. At high therapeutic doses, aspirin exerts an anti-inflammatory effect independent of the inhibition of prostaglandin synthesis; at lower therapeutic doses, aspirin enhances osteogenic differentiation. Aspirin at two concentrations was applied sequentially to modulate the activity of hMSCs at the initial stage and stimulate their osteogenic differentiation after the inflammatory stage. Culture of aspirin-treated hMSCs on PS-PEP promoted their osteogenic differentiation. Taken together, our data suggest that the combination of aspirin treatment of hMSCs and an osteogenic BFP-1 peptide-decorated substrate could significantly enhance immunomodulation and osteogenic differentiation. Future work in this field will involve *in vivo* transplantation of aspirin-treated hMSCs on an osteogenic BFP-1 peptide-decorated substrate to study bone tissue formation. This strategy has considerable potential for bone tissue engineering.

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