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LPS-Induced Inflammation Potentiates Dental Pulp Stem Cell Odontogenic Differentiation Through C5aR and p38

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Abstract

Aim: Inflammation is a complex host response to harmful infection or injury, and it seems to play a crucial role in tissue regeneration both positively and negatively. We have previously demonstrated that the activation of the complement C5a pathway affects dentin-pulp regeneration. However, limited information is available to understand the role of the complement C5a system related to inflammation-mediated dentinogenesis. The aim of this study was to determine the role of complement C5a receptor (C5aR) in regulating lipopolysaccharide (LPS)-induced odontogenic differentiation of dental pulp stem cells (DPSCs).

Material and Methods: Human DPSCs were subjected to LPS-stimulated odontogenic differentiation in dentinogenic media treated with the C5aR agonist and antagonist. A putative downstream pathway of the C5aR was examined using a p38 mitogen-activated protein kinase (p38) inhibitor (SB203580).

Results: Our data demonstrated that inflammation induced by LPS treatment potentiated DPSC odontogenic differentiation and this is C5aR dependent. C5aR signaling controlled the LPS-stimulated dentinogenesis by regulating the expression of odontogenic lineage markers like dentin sialophosphoprotein (DSPP) and dentin matrix protein 1 (DMP-1). Moreover, LPS treatment increased the total p38, and the active form of p38 expression, and treatment with the SB203580 abolished the LPS-induced DSPP and DMP-1 increase.

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Author contributions

Kim JH and Irfan M contributed to conception, design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript. Kim JH conducted all PCR experiments and also contributed to data acquisition, analysis of the experiment. MA Hossain and Shin S performed the control differentiation experiment. Chung S and George A designed the original concept and contributed to data acquisition and interpretation and financially supported the project.

Disclosure statement

The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article. I affirm that I/We have no financial affiliation, or involvement with any commercial organization with a direct financial interest in the subject or materials discussed in this manuscript, nor have any such arrangements existed.

Conclusions: These data suggest a significant role of C5aR and its putative downstream molecule p38 in the LPS-induced odontogenic DPSCs differentiation. This study highlights the regulatory pathway of complement C5aR/p38 and a possible therapeutic approach for improving the efficiency of dentin regeneration during inflammation.

Keywords

Inflammation; LPS; C5a receptor; p38; Dentinogenesis; DPSC

Introduction

Dental caries is an infectious disease that affects the oral tissue environment caused by the penetration of oral bacteria into the enamel and dentin. Extension of the bacterial caries infection to dentin leads to migration and differentiation of dental pulp stem cells (DPSCs) to the injured site and performs a regeneration process of 'tertiary' dentin formation [1]. Current clinical therapies (e.g., pulp capping) aim to promote dentin regeneration, yet do not address the underlying inflammatory process that may thwart odontoblast differentiation. Thus, therapeutic dentin regeneration remains elusive.

Inflammation is required to remove dead cells and debris from the site of injury and facilitates the repair process [2]. Inflammatory cells recruited to an injury site release cytokines and growth factors, notably TNF-α and ROS, at low levels to promote reparative dentinogenesis [3,4]. During inflammation, the complement system can promptly react to the stimuli and activate adequate host defense reactions. The complement fragment C5a is crucial in innate immunity, and triggers mast cell degranulation, releasing proinflammatory cytokines [5]. As a powerful pro-inflammatory mediator and chemotactic factor, it interacts with the G-coupled protein receptor (GPCR), and C5a receptor (C5aR) [6,7]. This binding process is critically engaged not only in inflammatory conditions but also in the development, homeostasis, and regeneration of several tissues [8,9]. Interestingly, we have recently demonstrated that the complement C5a system, which is an important mediator of inflammation and tissue regeneration, is activated in caries and regeneration processes. In response to the dental pulp inflammation, activation of C5a and the binding process of C5aR are involved in important early steps in dentin pulp regeneration by migration of the immune cells and pulp stem cells to the injured area [10,11].

We demonstrated that activation of C5aR is involved in the pulp nerve growth beneath carious injury [11–13]. Furthermore, we identified the role of C5aR in DPSC in odontoblastic differentiation and in vivo dentin formation [14,15]. Clinical dentinogenesis associated with caries occurs in an inflammatory environment. Understanding inflammatory responses on DPSCs is crucial to understand dentin and pulp regeneration. However, there is limited information available on the role of inflammation and C5aR in odontoblastic DPSC differentiation and dentinogenesis. Therefore, in this study, we sought to identify a significant role of the C5aR and its putative downstream molecule p38 in LPS (lipopolysaccharide)-induced dentinogenesis.

Methods

Cell culture and differentiation

Human DPSCs were commercially purchased from Lonza, Pharma and Biotech (Cat. # PT-5025), guaranteed through 10 population doublings, to express CD105, CD166, CD29, CD90, and CD73, and to not express CD34, CD45, and CD133. Cells between the 2nd and 4th passage were used in the experiment at 1×10^4 cells/well concentration. They were cultured in αMEM containing 10% fetal bovine serum (FBS) 1% L-glutamine and antimycotic/antibiotic at 37˚C and 5% CO_2 in regular/osteogenic media for 4 days. After that, they were cultured in osteogenic media of αMEM supplemented with 20% FBS, 1% L-glutamine and antimycotic/antibiotic, 50 μg/mL ascorbic acid, 10 mM β-glycerophosphate and 10 nM dexamethasone for 21 days. They were also treated 1–2 times of LPS (1μg/mL) depending on the experiment every three days and C5aR agonist (20 nM) or C5aR antagonist (10 nM), W54011, were treated every three days until day 24. The medium was changed before treatments. All experiments were conducted according to Institutional Animal Care and Use Policy and approved by the IRB Protocol Committee at the University of Illinois at Chicago (Permit number: 19–022).

Chemicals and reagents

The C5aR agonist was purchased from Anaspec (Fremont, CA, USA), the C5aR antagonist (W54011) from Calbiochem (San Diego, CA, USA), and the p38 inhibitor (SB203580) from Fisher Scientific. MEM-alpha, PBS, fetal bovine serum, L-glutamine, and Antibiotic-Antimycotic were procured from Gibco™ Fisher Scientific (Waltham, MA, USA). 12 mm Poly-D-Lysine coated (BioCoat™) round German glass coverslips slips were purchased from Corning™ Fisher Scientific (Waltham, MA, USA). Various antibodies were procured: anti-C5a receptor (Proteintech, ST. Louis, MO, USA), mouse anti-DMP-1 (R&D System/ Sigma, ST. Louis, MO, USA), mouse anti-DSPP (Santa Cruz, Dallas, Texas, USA). p38 inhibitor: 10 μM (AdipoGen) AG-CR1–0030-M005.

Real-time PCR (qPCR)

DPSCs were cultured in 6 wells plate at 5×10^4 cells/well according to the differentiation protocol (Figure 1) with the treatment of the C5aR agonist, W54011, or SB203580. Total mRNA was extracted with 0.8 ml Trizol (Invitrogen, Waltham, MA, USA) and analyzed using the Fisher Scientific NanoDrop 2000 device. The cDNA samples were analyzed using the Applied Biosystems SYBR green reagent system according to the manufacture's protocol. Primer sequences are as follows. hGAPDH; forward: 5′-GGC ATC CAC TGT GGT CAT GAG-3′, reverse: 5′-TGC ACC ACC AAC TGC TTA GC-3′, hDSPP; forward: 5′-CTG TTG GGA AGA GCC AAG ATA AG-3′, reverse: 5′-CCA AGA TCA TTC CAT GTT GTC CT-3′, hDMP-1; forward: 5′-CAC TCA AGA TTC AGG TGG CAG-3′, reverse: 5′-TCT GAG ATG CGA GAC TTC CTA AA-3′.

Immunofluorescence staining

Differentiating or differentiated DPSCs were fixed and permeabilized as previously described [14]. Subsequently, cells were incubated overnight with mouse anti-DMP-1

(1:1000), mouse anti-DSPP (1:500), anti-p38 (1:500), or anti-pp38 (1:500). Later, the cells were treated with secondary antibody for 3 hours with a mix of Alexa Fluor-594 anti-mouse IgG, Alexa Fluor-488 anti-rabbit IgG (1 μg/mL) and/or DAPI (2 μg/mL). The coverslips were mounted, and images were taken using a Leika microscope. Fluorescence density was quantified using MatLab software and values were analyzed for statistical significance by SAS 9.4.

Alkaline Phosphatase Activity (ALP)

ALP activity was analyzed as an indicator of enzymatic activity consistent with mineralization. DPSCs were cultured and fixed as previously described after 14 days. The cells were washed with DI water and treated with 1ml of AP color reagent in each well composed of 1% of AP color reagent A (contains nitroblue tetrazolium in aqueous dimethylformamide [DMF] and magnesium chloride), 1% of color reagent B (5-bromo-4-chloro-3-indolylphosphate in DMF), and 98% of AP color development buffer (25X liquid concentrate). The plates were incubated with a gentle shake for 2 hours at RT. Then, cells were washed with DI water and dried completely and analyzed with a Leika DMi1 microscope. Colorimetric AP Conjugate Substrate Kit (Bio-Rad #1706432) was used to identify the enzymatic activity of ECM mineralization in DPSC.

Alizarin Red Staining (ARS)

The 6-well plates were wash cells two times with DI water and fixed with 4% formalin for an hour at RT. Then cells were washed with DI water two times and applied 1ml of 40mM ARS per well provided by ScienCell (#8678). After an hour with a gentle shake, the plate was washed with DI water three times, and removed the excessed water. The cells were inspected by using a phase microscope and took images with Leika DMi1.

DMP-1 enzyme-linked immunosorbent assay (ELISA)

Supernatants were collected from the DPSCs odontoblastic differentiation which was incubated with various above-mentioned treatments. The DMP-1 ELISA kit was used for the experiment according to the manufacturer's protocol (R&D Systems). A standard curve was used to form based on the standards and sample values and normalized according to the duplicated test samples at increasing concentrations.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 6.0 (GraphPad Software, USA). A comparison between the two groups was made using the unpaired Student's t-test. For multiple comparisons, data were analyzed using the one-way ANOVA followed by the Tukey test. Detailed statistics for each experiment were shown in the figure legends. Data are presented as mean ± SD. Differences among means with $p < 0.05$ were considered significant.

Results

LPS treatment increased DSPP and DMP-1 expression during DPSC odontoblastic differentiation.

Odontoblastic differentiation of DPSCs under a carious injury occurs in an inflammatory context [1]. To identify the effect of inflammation in dentinogenesis, and the involvement of C5aR in this process, we utilized lipopolysaccharide (LPS) which is one of the most potent single inducer of inflammation[16], and C5aR agonist/antagonist during 17-days of odontoblastic differentiation. The human DPSCs were cultured using a regular growth medium for 3 days and then in dentinogenic media for 3 weeks. LPS was applied to induce the inflammation at day 4 and 7, and cells were treated with a C5aR agonist or antagonist every 3 days starting from day 4 according to a timeline scheme (for details, see Figure 1A). Treatment groups were: DPSCs in dentinogenic media (control), LPS, LPS + C5aR agonist, and LPS + C5aR antagonist (W54011) treatments were compared to identify the expression of the odontogenic lineage markers, such as dentin sialophosphoprotein (DSPP) and dentin matrix protein 1 (DMP-1) by immunocytochemistry (Figure 1). DSPP and DMP-1 play essential roles in hard tissue development and are positive regulators of dentin mineralization [17]. LPS treatment significantly enhanced the expression of DSPP (267.5 ± 22.1, $p < 0.001$) and DMP-1 (314 ± 52.8, $p < 0.001$) confirmed by the fluorescent intensity from immunocytochemistry compared with the control (Figure 2. A, B). Also, mRNA expression of DSPP was increased that of DMP-1 was increased slightly (Figure 2 C, D). These data suggest that inflammation induced by LPS treatment has a positive role in the odontoblastic differentiation of DPSCs.

C5aR modulates LPS-mediated enhanced DSPP and DMP-1 expression during DPSC odontoblastic differentiation.

Since our previous studies suggest that C5aR expression/activation is dependent on inflammatory contexts [14], we next investigated the role of C5aR in LPS-mediated DPSC odontoblastic differentiation. Co-stimulation of LPS with C5aR agonist significantly increased the expression of DSPP (408.5 ± 30.2; $p < 0.05$) and DMP-1 (606.7 ± 47; $p < 0.01$) compared with alone LPS alone treated group, while C5aR-antagonist abolished this effect ($p < 0.05$) (Figure 2. A, B). Our mRNA analyses support the above immunocytochemical data (Figure 2. C, D). The mRNA expressions of DSPP and DMP-1 were compared with three treatment groups of LPS-stimulated odontogenic differentiation of DPSCs at day 10 (Figure 2 C, D). Data represent a significant increase of DSPP expression in the LPS treatment and LPS+C5aR agonist groups compared to the control. The mRNA expression of DSPP was decreased in the W54011 treatment group compared to the LPS treatment group and the control (Control vs LPS; 0.5522 ± 0.13, Control vs LPS+C5aR agonist; 1.563 ± 0.4638, Control vs LPS+W54011 -0.200 ± 0.026, LPS+C5aR agonist vs LPS+W54011; -1.763 ± 0.4645, LPS vs LPS+W54011; -0.7522 ± 0.1351, $p < 0.05$; unpaired Students t-test, difference between means ± SD). The mRNA expression of DMP-1 was slightly increased in the LPS treatment group compared to the Control (0.4958 ± 0.5071, Figure 2B). However, there was a significant decrease in the LPS+C5aR agonist compared to the LPS+W54011 (-0.5756 ± 0.2050, Figure 2B). These results demonstrate

that the complement C5a receptor modulates LPS-induced odontogenic differentiation of DPSCs by regulating DSPP and DMP-1.

p38 expression/activation is increased in DPSCs following LPS treatment.

We have previously demonstrated that p38 modulation enhances the odontoblastic differentiation of DPSCs [18]. Given that p38 could be one of the downstream pathways of complement C5a [19] and our preliminary data suggest that C5aR-mediated BDNF control in pulp fibroblasts is p38 dependent, we next investigated the role of p38 in LPS-mediated dentinogenesis. To identify the expression of p38 in LPS-stimulated DPSCs odontogenic differentiation, immunocytochemistry was used to confirm the protein expression level in the early stage of LPS-induced DPSCs at day 4. The cells were treated with LPS at day 4, the immunopositivity of p38 was compared to control cells (Figure 3. A–F) and quantified the fluorescence intensity (Figure 3. G). Also, co-localization of phosphorylated p38 (pp38) and C5aR in LPS-treated cells was observed and compared with control (Figure 3. H–O) and analyzed the immunopositivity (Figure 3. P). Our data suggest that p38 and pp38 is expressed and activated in the LPS-stimulated DPSCs and co-localized with an increased C5aR expression.

p38 regulates LPS-mediated DSPP and DMP-1 expression.

To demonstrate the specific role of p38 in odontogenic DPSCs differentiation under LPS-treated conditions, cells were treated with a LPS and p38 inhibitor (SB203580) at day 4 and 7 (Figure 4A). The conditions were the same as the above-mentioned experimental conditions except for the treatment with SB203580 instead of C5aR-agonist and antagonist. We examined the expression of odontogenic differentiation markers, DSPP and DMP-1, in LPS-induced DPCSs differentiation at day 10 (Figure 4. B–P). LPS-stimulated DPSCs showed higher expression of DSPP (327.7 ± 30, $p < 0.001$) and DMP-1 (295.8 ± 29, $p < 0.001$) while co-stimulation of LPS and SB203580 significantly decreased the fluorescent intensity of DSPP (180.2 ± 30.3, $p < 0.05$) and DMP-1 (126.7 ± 26, $p < 0.05$) compared to LPS treated group (Figure 5. A, B). Also, the mRNA expression of DSPP and DMP-1 was slightly decreased even though there is no significant statistical difference. (Figure 5. C, D. Control vs LPS, Control vs LPS+ SB203580, LPS vs LPS+ SB203580; NS, $p < 0.001$, Unpaired Student t-test). Our ELISA data confirmed that the DMP-1 production was significantly increased during in the LPS-treated DPSCs, and LPS + C5aR agonist-treated DPSCs in the supernatant. On the other hand, W54011 and SB203580 treatment reversed the DMP-1 production showing a similar trend observed in ICC and qPCR data (Figure 6. G). Taken together, our data suggest the role of p38 in LPS-mediated odontoblastic DPSC differentiation.

Mineralization activity of LPS-induced DPSCs odontoblastic differentiation through C5aR and p38.

The mineralization activity of DPSCs in different treatment groups was examined through alizarin red staining (ARS), alkaline phosphate (ALP) and ELISA. ARS showed an visual increase in the calcium deposition of the LPS-treated DPSCs compared to the control DPSCs (Figure 6. A vs B), and LPS+ C5aR agonist-treated DPSCs (Figure 6. A vs D). Treatment of LPS + SB203580 or LPS + W54011 significantly decreased the calcium

deposition compared to the treatment of LPS and this confirmed the effect of SB203580 and W54011 in the mineralization activity (Figure 6. B vs C, E). The image quantification of positively stained area (%) was analyzed which shows significant differences between each group (Figure 6. F). ALP activity was used to confirm and evaluate the differentiation status of mineralized DPSCs matrix through p38. The images of ALP activity show an optical increase of mineralization in the LPS treatment group compared to the control and a decrease in the LPS + SB203580 (Figure 6. H vs I, J). The image quantification of ARS and ALP activity showed same data patterns and this was confirmed by DMP-1 ELISA (Figure 6. G). The results supported that LPS-induced DPSCs odontoblastic differentiation through C5aR and p38 increase the mineralization activity and DMP-1 protein expression of ELISA.

Discussion

In this study, we identified the roles of inflammation and C5aR modulation in promoting the odontoblast differentiation of DPSCs. The application of LPS to DPSCs led to inflammation-induced dentinogenesis. Our data indicate that LPS potentiated DSPP and DMP-1 expression and this effect was C5aR dependent. We also sought the role of p38 in a related pathway of C5aR during LPS-induced DPSCs odontogenic differentiation. SB203580 treatment also abolished the increase of LPS-mediated DSPP and DMP-1 expression suggesting its essential role in this process.

The complement system, the first line of defense in the immune system is another critical factor of the inflammatory process besides various cytokines. Complements are activated by pathogen infection, leading to inflammation, opsonization, phagocytosis, and destruction of the pathogen ultimately resulting in the activation of the adaptive immune response [20]. Beyond its role in immunity, the complement system participates in tissue regeneration [21]. The activation of the complement system during inflammation affects dentin-pulp regeneration by migrating pulp progenitors to the injury site and promoting pulp nerve growth beneath a carious injury [22].

Our results demonstrated the expression of the odontogenic lineage cell markers in LPS-stimulated DPSCs differentiation via complement C5a receptor. The application of LPS during the differentiation activated the complement C5a receptor. During inflammation, the molecular expressions of DSPP and DMP-1 were measured by comparing the control, LPS, LPS + C5aR agonist, and LPS + W54011 treatment groups (Figure 1, 2). The fluorescent intensities of both DSPP and DMP-1 were significantly changed in the agonist and antagonist-treated cells. The result of the mRNA expression level of DSPP corroborated with the protein expression, while an inhibitory trend can be seen in the DMP-1 expression level. These data are consistent the result in our recent publication [23] that demonstrated the role of the C5a receptor and inflammation in modulating key nerve regeneration factors such as BDNF and NGF.

DSPP and DMP-1 share many similarities in both their gene and protein structures. They play on important role in hard tissue development and are considered positive regulators of dentin mineralization [24]. Despite the similarities between them, they have discrete biochemical differences. They induce different signaling at different timepoints throughout

dentinogenesis. DSPP has a potential role in cell signaling and functions as an inducer of mineralization in the extracellular matrix [25]. DMP-1 is expressed during the early to late stages of odontogenesis using the Col1a promoter and during the late stage of odontogenesis using the DSPP promoter [26]. It is critical to examine the protein and mRNA expressions in early, mid, and late stages of dentinogenesis. However, the regulatory mechanism for the gene expression of DSPP and DMP-1 is not fully understood especially in the inflammation context [17]. In our data, the expression of DSPP and DMP1 during stimulation of LPS and p38 inhibitor revealed slight, but not significant changes. This could be attributed to the different expression stages or application of the inflammatory components.

According to a recent article, LPS was shown to promote the odontoblast differentiation of DPSC via TLR4, ERK, and p38 MAPK signaling pathways [27]. In our study, we demonstrated that LPS-induced odontogenic differentiation of DPSC is modulated by C5aR. In addition, we sought to identify the involvement of possible downstream pathways of complement C5a during LPS-induced odontogenic differentiation using immunocytochemistry and mRNA expression. It was suggested that one of the downstream pathways of complement C5a is p38, which is crucial for C5a-induced chemotactic cell migration [28]. It is well known that the p38 has multiple roles in signaling mechanisms, tissue hemostasis, and proliferation, differentiation, survival, and the migration of specific cell types [29–31]. It inhibits the biosynthesis of inflammatory cytokines interleukin-1 (IL-1) and tumor necrosis factor (TNF) in LPS-stimulated monocytes [32]. Activation of p38 has been identified in response to tooth morphogenesis and enamel production [33]. Furthermore, p38 signaling is known to be involved in DMPs stimulated osteoblast differentiation [34]. However, there is limited information available on the complement system related to LPS-induced dentinogenesis. Many studies on p38 activation following inflammation show short-lived activation and expression between 8 and 17 days [35,36]. To determine the expression levels of p38 in the LPS-induced odontogenic differentiation of DPSCs, we examined the expression of the early and middle stages of LPS-induced DPSCs by comparing the control, LPS, and LPS + SB203580 treatment groups. The molecular expressions of the p38 and phosphorylated p38 were increased in the LPS-stimulated DPSCs (Figure 3, 4). We identified the involvement of p38 during the mid-stage of the odontogenic differentiation by using a p38 inhibitor (Figure 4). Significant differences were between the fluorescence intensities of DSPP and DMP-1. The mRNA expression trends of DSPP and DMP-1 corresponded to the intensities. Since it was in the middle stage of the odontogenic differentiation, the difference in the expression level might not be significant.

Our ARS and ALP data confirmed the mineralization activity in LPS-induced odontoblastic DPSC differentiation through C5aR and p38. ARS detected and visualized calcium deposits and the mineralization of the ECM was confirmed by imaging quantification. The cellular ALP activity was measured colorimetrically and showed metastasis of mineralization function in ECM. The cellular ALP is used to evaluate the odontogenic differentiation of DPSC which may affect the deposition of the mineralized matrix. The present study focused on the enzymatic activity of ALP in different exogenic proteins treatment and its contribution to mineralization in odontogenic differentiation cultures.

Taken together, the present work confirms that activation of C5aR during inflammation has a positive regulatory role in DPSC odontogenic differentiation. We found that activation of complement C5aR increases DSPP and DMP-1 expression during LPS-induced dentinogenesis. Moreover, the results demonstrate the downstream pathway of the complement system, and importantly, p38 was identified to be involved in the early and mid-stage of LPS-induced dentinogenesis. PCR and immunocytochemistry successfully detected mRNA and protein expression of odontogenic lineage cell markers. In conclusion, our study shows that LPS-stimulated C5aR activation induced resulted in better odontogenic differentiation of DPSCs. Targeting C5aR during an inflammatory insult may provide novel therapeutic management strategies for tertiary reparative dentin regeneration.

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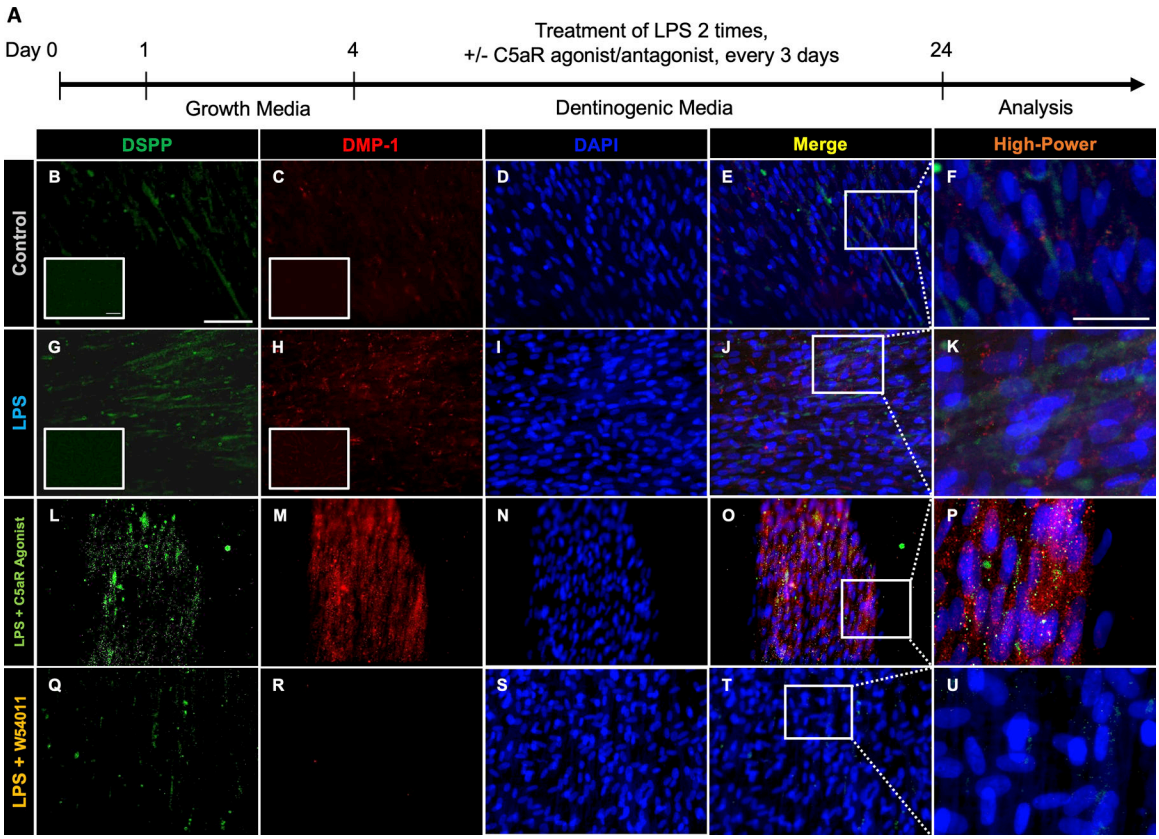

Figure 1. Complement C5a receptor-mediated DSPP and DMP-1 expression in LPS-stimulated DPSCs.

DPSCs were cultured in a regular growth media for 3 days. Subsequently, cells were cultured in dentinogenic media and treated with C5aR agonist or antagonist (W54011) from the differentiation day 4 to day 24. The culture media was replaced every 3 days. Representative images of differentiating DPSCs at day 4 (A) Schematic representation of LPS-stimulated differentiation of DPSCs. (B) The expression of DSPP (Green) in control DPSCs at day 24. A small white box indicated the expression of DSPP for undifferentiated DPSCs at day 4. (C) The expression of DMP-1 (Red) in Control DPSCs. A small white box indicated the expression of DMP-1 for undifferentiated DPSCs at day 4. (D) The expression of DAPI (Blue) in Control DPSCs. (E) Merged images of overlap between DSPP, DMP-1, and DAPI (B, C, D). (F) Higher magnification of (E) showing co-localized in the cytoplasm of DPSCs. (G) The expression of DSPP (Green) in LPS-treated DPSCs at day 24. A small white box indicated the expression of DSPP for undifferentiated DPSCs at day 4. (H) The expression of DMP-1 (Red) in LPS-treated DPSCs. A small white box indicated the expression of DMP-1 for undifferentiated DPSCs at day 4. (I) The expression of DAPI (Blue) in LPS-treated DPSCs. (J) Merged images of overlap between DSPP, DMP-1, and DAPI (G, H, I). (K) Higher magnification of (J) showing co-localized in the cytoplasm of DPSCs. (L) The expression of DSPP (Green) in LPS+C5aR-agonist DPSCs at day 24. (M) The expression of DMP-1 (Red) in LPS+C5aR-agonist DPSCs. (N) The expression of DAPI (Blue) in LPS+C5aR-agonist DPSCs. (O) Merged images of overlap between DSPP, DMP-1, and DAPI (L, M, N). (P) Higher magnification of (O) showing co-localized in the

cytoplasm of DPSCs. (Q) The expression of DSPP (Green) in LPS+W54011-treated DPSCs at day 24. (R) The expression of DMP-1 (Red) in LPS+W54011-treated DPSCs. (S) The expression of DAPI (Blue) in LLPS+W54011-treated DPSCs. (T) Merged images of overlap between DSPP, DMP-1, and DAPI (Q, R, S). (U) Higher magnification of (T) showing co-localized in the cytoplasm of DPSCs. Scale bar: B & Small White box for B = 200 μm; F= 50 μm.

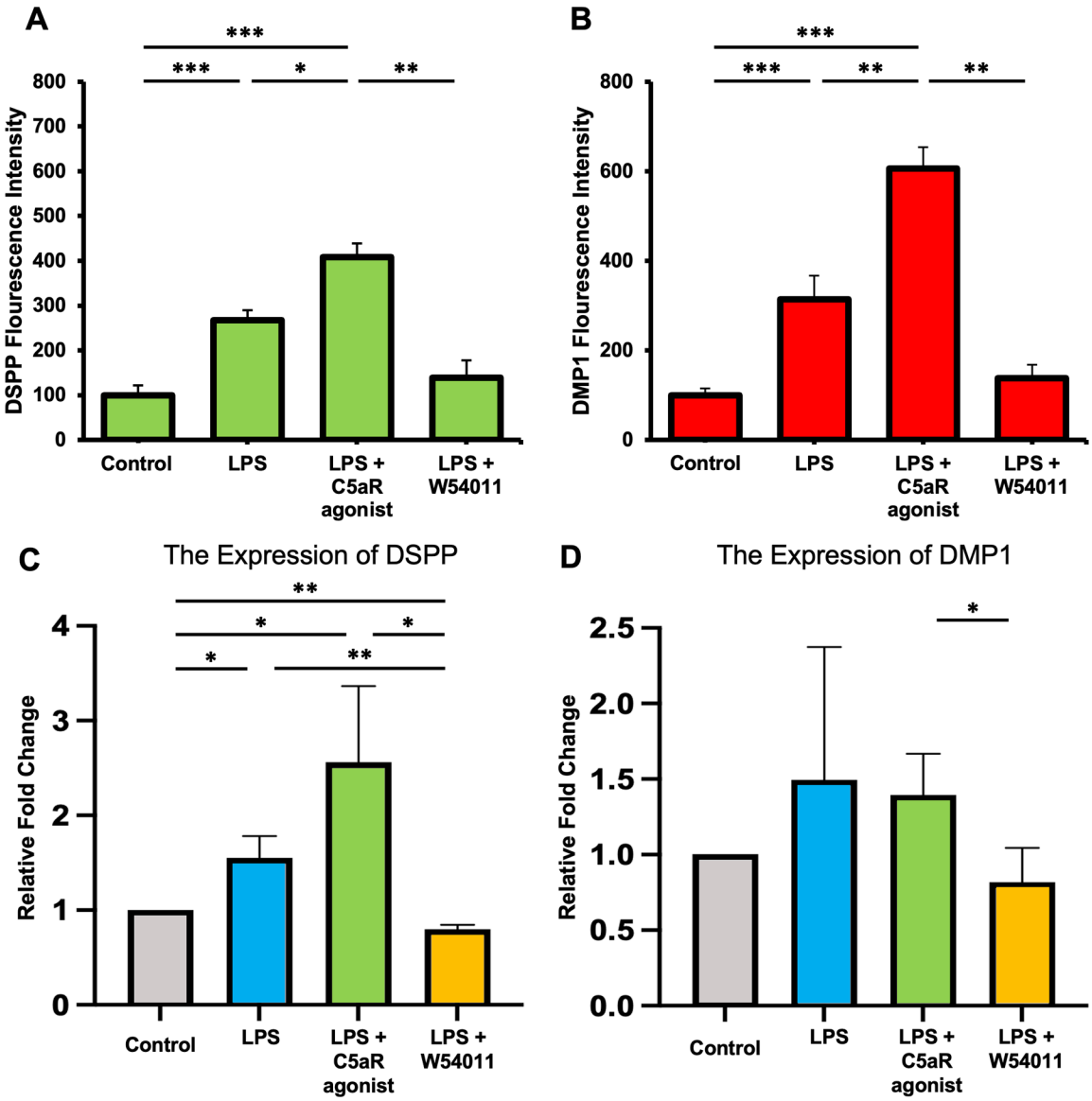

Figure 2. The analysis of immunoreactivity and mRNA expression of DSPP and DMP-1 in LPS-induced odontogenic differentiation of DPSCs mediated by C5aR.

(A) The quantification of DSPP fluorescence intensity for LPS-stimulated DPSCs mediated by C5aR (from Figure 1. B, G, L, Q). **(B)** The quantification of DMP-1 fluorescence intensity for LPS-stimulated DPSCs mediated by C5aR (from Figure 1. C, H, M, R). (C) Quantitative PCR (qPCR) analysis of DSPP normalized against GAPDH in LPS-induced odontogenic differentiation of DPSCs at day 10. The significant difference compared with the control group (as represented by value 1) and the application of LPS, LPS+C5aR agonist, and LPS+W54011. (D) qPCR analysis of DMP-1 normalized against GAPDH with various treatment groups. Values represent mean ± SD among n = 3 individual experiments performed on independent days; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared with control.

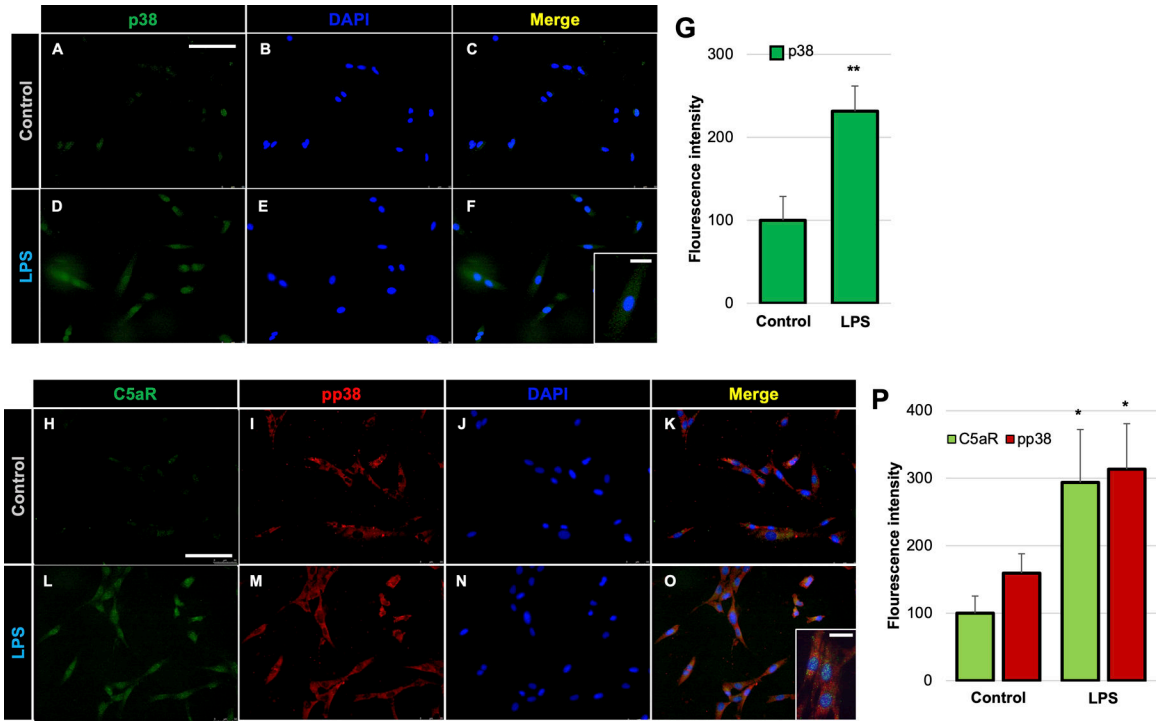

Figure 3. Expression of pp38 and p38 in the early stage of LPS-stimulated DPSCs.

(A) Immunofluorescent image of p38 (Green) in control DPSCs at day 4. (B) DAPI (Blue) in resting control DPSCs. (C) Merged images showed an overlap between (A) and (B). (D) Immunofluorescent image of p38 (Green) in LPS-treated DPSCs at day 4. (E) DAPI (Blue) in LPS-treated DPSCs. (F) Merged images showed an overlap between (D) and (E). (G) Fluorescence intensity of p38 compared with the control and LPS treatment. (H) The expression of C5aR (Green) in the resting control DPSCs at day 4. (I) Anti-pp38 MAPK (Red) was homogenously expressed in the cytoplasm of DPSCs, and its expression was increased on LPS stimulation. (J) The expression of DAPI (Blue) in the resting control DPSCs. (K) Merged images showed an overlap between (H, I, J). (L) The expression of C5aR (Green) in LPS-stimulated DPSCs at day 4. (M) The expression of Anti-pp38 MAPK (Red) in LPS-stimulated DPSCs. (N) The expression of DAPI (Blue) in LPS-stimulated DPSCs. (O) Merged images showed an overlap between (L, M, N). Scale bar in (A, H): 100 μm. Magnified image (white box of F, O) scale bar: 50 μm. (P) Fluorescence intensity of C5aR and pp38 compared with the control and LPS treatment (* $p < 0.05$ and ** $p < 0.01$).

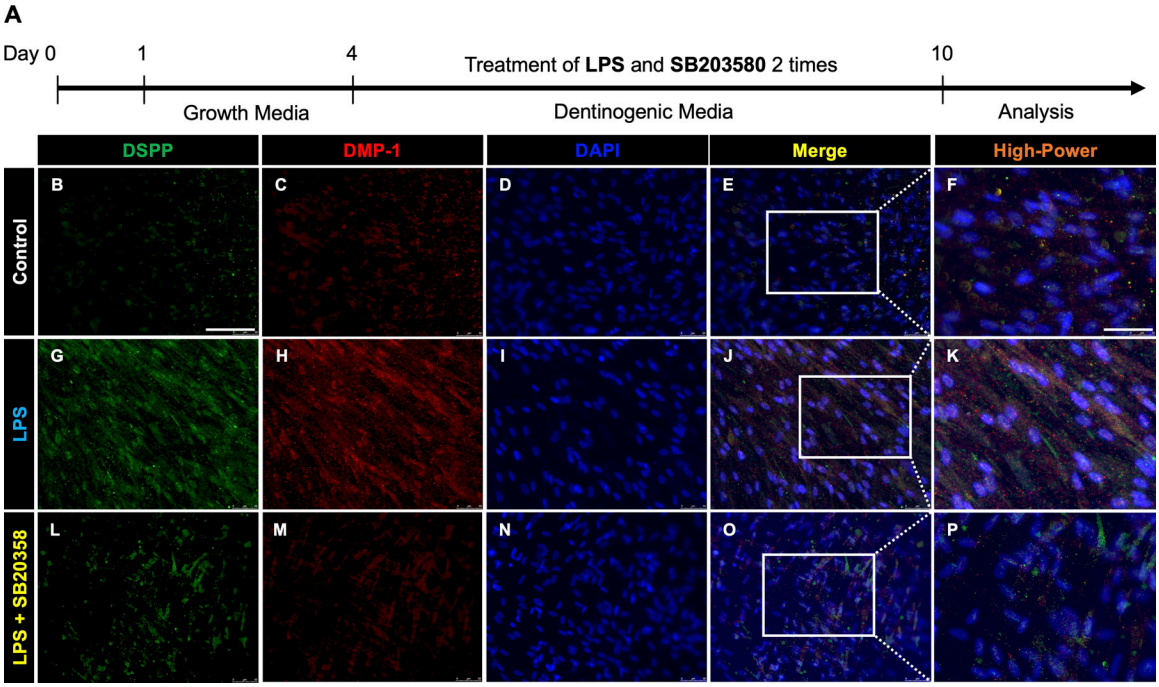

Figure 4. Effect of p38 inhibitor (SB203580) on the expression of DSPP and DMP-1 in LPS-stimulated DPSCs.

(A) Schematic representation of LPS-stimulated differentiation of DPSCs. (B) The immunofluorescence images of DSPP expression (Green) in control DPSCs. (C) DMP-1 (Red) expression of control DPSCs. (D) DAPI (Blue) staining in control DPSCs. (E) The merged image showed overlap between DSPP, DMP1, and DAPI from (B, C, D). (F) Higher magnification of white box from (E) showing co-localized in the cytoplasm of DPSCs. (G) DSPP (Green) expression in LPS-treated DPSCs at day 10. (H) DMP-1 (Red) expression in LPS-treated DPSCs. (I) DAPI (Blue). (J) Merged images of overlap between DSPP, DMP-1, and DAPI (G, H, I). (K) Higher magnification of white box from (J) showing co-localized in the cytoplasm of DPSCs. (L) DSPP (Green) expression in LPS+SB203580-treated DPSCs at day 10. (M) DMP-1 (Red) expression in LPS+SB203580-treated DPSCs. (N) DAPI (Blue). (O) Merged images of overlap between DSPP, DMP-1, and DAPI (L, M, N). (P) Higher magnification of white box from (O). Scale bar: B = 100 μm; F = 50 μm.

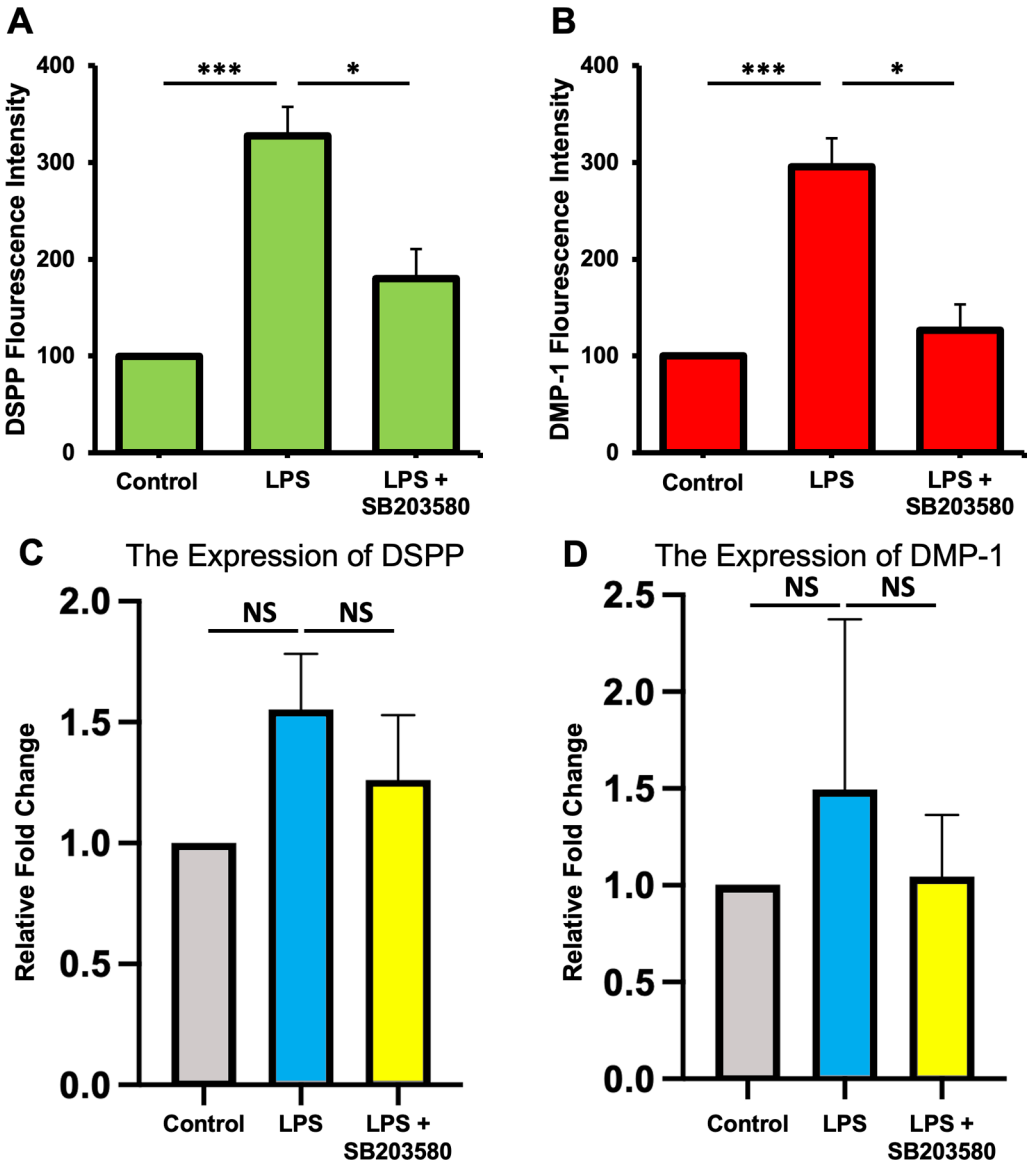

Figure 5. Statistical analysis of p38 inhibitor on expression of DSPP and DMP-1 in LPS-stimulated DPSCs.

(A) The quantification of DSPP fluorescence intensity for LPS-stimulated DPSCs treated with SB203580 (From Figure 4. B, G, L). (B) DMP-1 fluorescence intensity quantification for LPS-stimulated DPSCs treated with SB203580 (from Figure 1. C, H, M). (C) qPCR analysis of DSPP normalized against GAPDH in LPS-induced odontogenic differentiation of DPSCs with SB203580 at day 10. The treatment group of LPS, LPS+C5aR agonist, and LPS+W54011 were compared. (D) qPCR analysis of DMP-1 normalized against GAPDH with various treatment groups. Values represent mean ± SD among n = 3 individual experiments performed on independent days: * $p < 0.05$ and *** $p < 0.001$.

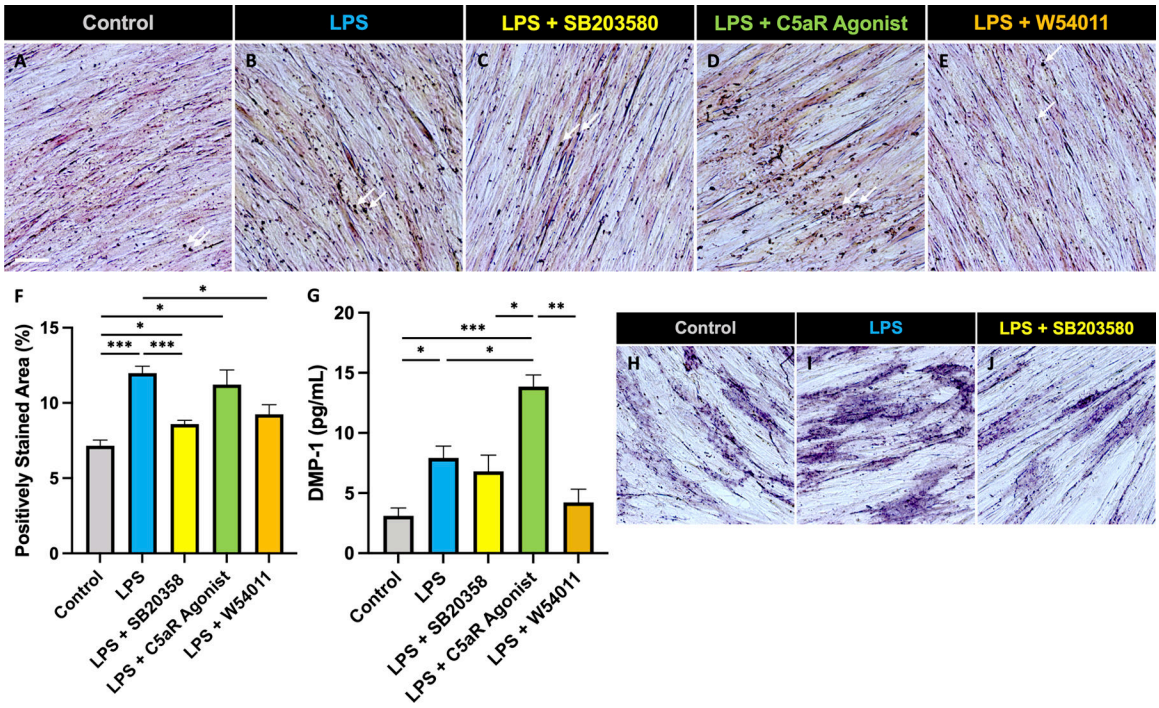

Figure 6. Effects of LPS-induced DPSCs odontoblastic differentiation through C5aR and p38 in mineralization assays and DMP-1 expression.

(A) Alizarin Red Staining (ARS) of resting control group showing mineralization for 17-days. White arrows indicate the presence of calcium deposition. (B) LPS-treated DPSCs. (C) LPS and SB203580 treated DPSCs. (D) LPS and C5aR agonist treated DPSC. (E) LPS and W54011 treated group. White scale bar: 50 μm. (F) Image quantification of the ARS (n= 4; *p < 0.05, **p < 0.01, and ***p < 0.001). **(G)** DMP-1 ELISA for DPSCs odontoblastic differentiation with various treatments such as control, LPS, LPS+C5aR-Agonist, LPS+W54011, LPS+ SB203580. (Control vs LPS+W54011, LPS + SB203580: NS. LPS vs LPS+W54011, LPS + SB203580: NS. LPS+W54011 vs LPS + SB203580: NS.) (*p < 0.05, **p < 0.01, and ***p < 0.001).