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Published in:
International Endodontic Journal

Publication date:
2020

Document Version
Peer reviewed version

INTERNATIONAL ENDODONTIC JOURNAL
The official journal of the British Endodontic Society and the European Society of Endodontology

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Journal:	*International Endodontic Journal*
Manuscript ID	IEJ-19-00560.R2
Wiley - Manuscript Type:	Original Scientific Article
Keywords:	Dental pulp stem cells, Toll-like receptor, TLR2 agonist, Lipoteichoic acid

Lipoteichoic acid stimulates the proliferation, migration and cytokine production of adult dental pulp stem cells without affecting osteogenic differentiation

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Running title: DPSCs and Lipoteichoic acid

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Abstract

Aim To model *in vitro* the contact between adult dental pulp stem cells (DPSCs) and lipoteichoic acid (LTA), a cell wall component expressed at the surface of most Gram-positive bacteria.

Methodology Human DPSCs obtained from impacted third molars were cultured and exposed to various concentrations of *S.aureus* LTA (0.1, 1.0 and 10.µg/mL). Effects of LTA on DPSCs proliferation and apoptosis were investigated by MTT assay and flow cytometry. Mineralization of DPSCs was evaluated by alizarin red staining assay. Migration was investigated by microphotographs of wound healing and Transwell migration assays. Reverse transcription polymerase chain reaction was used to examine the effects of LTA on p65 NF-κB translocation and TLR1, -2 or -6 regulation. Enzyme-linked immunosorbent assay was used to investigate LTA-stimulated DPSCs cytokine production. One-way or two-way ANOVA and Tukey post-hoc multiple comparison were used for statistical analysis

Results DPSCs expressed TLR1, -2 and -6 involved in the recognition of various forms of LTA or lipoproteins. Exposure to LTA did not up- or down- regulate the mRNAs of TLR1, -2 or -6 while LPS acted as a potent inducer of them [TLR1 ($P \leq 0.05$), TLR2 ($P \leq 0.001$) and TLR6 ($P \leq 0.001$)]. Translocation of p65 NF-κB to the nucleus was detected in LTA-stimulated cells, but to a lesser extent than LPS-stimulated DPSCs ($P \leq 0.001$). The viability of cells exposed to LTA was greater than unstimulated cells, which was attributed to an increased proliferation and not to less cell death [LTA 1µg/mL ($P \leq 0.001$) and 10 µg/mL ($P \leq 0.01$)]. For specific doses of LTA (1.0 µg/mL), adhesion of DPSCs to collagen matrix was disturbed ($P \leq 0.05$) and cells

enhanced their horizontal mobility ($P \leq 0.001$). LTA-stimulated DPSCs released IL-6 and IL-8 in a dose-dependent manner ($P \leq 0.0001$). At all concentrations investigated, LTA did not influence osteogenic/odontoblastic differentiation.

Conclusions Human DPSCs were able to sense the wall components of Gram-positive bacteria likely through TLR2 signaling. Consequently, cells modestly proliferated, increased their migratory behavior and contributed significantly to the local inflammatory response through cytokine release.

Introduction

During the initial phase, oral pathogens responsible for dental caries are essentially represented by Gram-positive and facultative anaerobic bacteria such as *Streptococcus mutans*. Through the production of various acids, these bacteria progressively drive the demineralization of enamel and dentine, to finally gain access to the pulp (Bowen & Koo 2011). Once in contact with living tissues, the primary response of pulp cells relies on pathogen recognition and inflammation (pulpitis), which helps at recruiting and activating the immune system (Yu & Abbott 2007). The rapid sensing of pathogens involves highly conserved molecular domains of the pathogens with specialized pattern-recognition receptors (PRRs) among which are the Toll-like receptors (TLRs), expressed by non-immune and immune cells. TLR activation initiates the production of pro-inflammatory cytokines and chemokines that lead to the recruitment of innate immune cells (Yu & Abbott 2007). Ten human TLRs have been identified and divided into major families (Roach *et al*. 2005): the TLR1 group including TLR1, TLR2, and TLR6, detecting lipopeptides; TLR4 sensing lipopolysaccharides (LPS); TLR3 for double-stranded RNA; TLR5 for flagellin; the TLR7 family, including TLR7, TLR8 and TLR9, able to detect nucleic acids and TLR10 likely also detecting dsRNA (Jiang *et al*. 2016). Gram-positive bacteria typically express lipoteichoic acid (LTA) in their cell wall where it serves as a surface-associated adhesion factor and a regulator of autolytic wall enzymes (Morath *et al.* 2005, Schneewind & Missiakas 2017). LTA is one of the natural agonists of TLR2. TLR2 can heterodimerize with either TLR1 or TLR6, to discriminate between diacyl (TLR1/2) and triacyl (TLR2/6) lipopeptides. Overall, TLR2 mediates the responses towards zymosan, peptidoglycan and various forms of lipoproteins or lipoteichoic acids.

Dental pulp stem cells (DPSCs) are a minority of resident pulp cells that remain quiescent *in vivo*. Following an injury or during an infection, they can respond quickly and migrate to the affected pulp site (Goldberg *et al*. 2008). By their differentiation properties into odontoblasts, they actively build reactionary dentine, while also being involved in the regulation of the pulpal immune response (Cooper *et al.* 2010). DPSCs isolated from human third molars and grown in laboratory conditions have a high proliferative capacity with self-renewal and multi-differentiation properties (Gronthos *et al*. 2000, Podtar *et al*. 2015). *In vitro*, DPSCs express TLRs 1-10 at differential levels, with TLR1, TLR2, TLR6

and TLR4 found in significant amounts, making them susceptible to sense bacterial surface lipopolysaccharide (LPS) or lipoteichoic acid (LTA) (Hahn & Liewehr 2007, Carrouel *et al.* 2007, Fawzy El-Sayed *et al.* 2016). In DPSCs, inflammatory conditions modulate the expression level of many of TLRs. For instance, upregulation of TLR1, -2, -4, -5, -8 while downregulation of TLR7, -9, -10 are described when the DPSCs are exposed to a cocktail of pro-inflammatory cytokines (Fawzy El-Sayed *et al.* 2016). Moreover, major intracellular adaptor molecules have been found activated once DPSCs were incubated with LPS. For example, MyD88 has a key role in transducing the signal to the nucleus and triggering downstream effects (He *et al.* 2014, Feng *et al.* 2018, He *et al.* 2013). No study has assessed LTA as a stimulus mimicking infectious conditions where pulp stem cells are faced with Gram-positive bacteria. LTA is released extracellularly in large quantities by cariogenic Gram-positive bacteria when high amounts of sucrose are available (Rølla *et al*. 1980) and could diffuse up to the pulp in case of a breakdown of the enamel and dentine barriers. The aim of this study was to investigate the effect of various concentrations of purified LTA on DPSC proliferation, migration, differentiation, cytokine production and related-TLRs expression. The null hypothesis of this study was that LTA has no effect on the profileration, migration, cytokine production and osteogenic differentiation of DPSCs.

Materials and methods

Isolation and culture of DPSCs

The procedure used to extract human healthy teeth and dental pulp tissue in this study was approved by the Ethics Committee of the Children's Hospital of Queen Fabiola, Free University of Brussels (CEH 24/16). Patients/parents provided their written informed consent.

Pulp tissue was obtained from impacted third molars collected from healthy subjects, 13–16 years of age (n=8). All specimens were free of carious lesions. The coronal pulp was retrieved, minced into small fragments before digestion in a solution of 3 mg/mL collagenase type I (Gibco Life Technologies, Carlsbad, CA, USA) and Alpha Minimum Essential Media (α-MEM, Gibco Life Technologies), for 1 h at 37 °C in 5% CO_2. Cell suspension was filtered through a 70µm cell strainer (Greiner Bio-One

GmbH, Frickenhausen, Germany) and pelleted. Dental pulp cells were seeded into 25 cm^2 cell culture flasks (Cellstar®, Greiner Bio-One GmbH) and cultured in α-MEM Medium (Gibco Life Technologies) supplemented with 10% foetal bovine serum (FBS, Sigma-Aldrich, St-Louis, MO, USA), 0.5% 10,000 mg/mL penicillin/streptomycin (Gibco Life Technologies), 1% 1x Glutamax (Gibco Life Technologies) at 37 °C in 5% CO_2. The medium was changed every 3 days. When the cells became sub-confluent, they were collected by trypnization (trypsine 0.05% + EDTA 1x, Gibco Life Technologies) and processed for subsequent passages. Only DPSCs from passage 2 to 4 were used in all experiments. Cells were plated overnight at a density varying in each experiment and then stimulated with a range of concentrations of LTA (0.1, 1.0 and 10.0 µg/mL) purified from *Staphylococcus aureus* (LTA-SA, Invivogen, San Diego, CA, USA) or with LPS (1µg/mL) from *E. coli O111:B4* (LPS-EB, Invivogen), added to culture medium. Unstimulated cells (LTA or LPS 0 µg/mL), used as controls, received medium-only stimulation. HEK293-Blue™-hTLR2 reporter cells (Invivogen, ref. hkb-htlr2) were used to validate *S. aureus*-derived LTA as TLR2 agonist, according to manufacturer's instructions (Suppl. Fig 1).

The medium was regularly tested for mycoplasm detection (MycoAlert™ PLUS Mycoplasma Detection Kit, Loanza, Walkersville, MD, USA). The endotoxin content was also analyzed using a *Limulus amoebocyte* lysate test (Pierce™ LAL Chromogenic Endotoxin Quantitation Kit, Thermo Fischer scientific, Waltham, MA, USA). Endotoxin content of the DPSC final cultures and in the stock solution of LTA were below 2.5 EU/mL (threshold equal approx. to 0.5ng/mL of endotoxin) and mycoplasma testing results confirmed that no contamination was ever detected.

For TLR2 inhibition experiment, DPSCs were co-incubated with 10µM of CU-CPT22 (Cat. No. 4884, Bio-Techne, Abingdon, UK) and various concentrations of LTA, for 24 hours. CU-CPT22 is a TLR2/1 heterodimer antagonist with the ability to inhibit TLR2-mediated proinflammatory induction.

Immunofluorescence

DPSCs were washed three times with PBS 0.01M before fixation with buffered paraformaldehyde 4% for 15 minutes at room temperature. DPSCs were washed three times and kept in PBS at 4°C until use. DPSCs were permeabilized 15 minutes with 0.1% Triton X-100 in PBS and washed out with PBS. Coverslips were then incubated overnight at 4°C with primary antibodies (mouse IgG1 anti-human CD44, ref. H4C4, DSHB, 1:10; rabbit mAb anti-human Vimentin, ref. D21H3, 1:100, Cell Signaling Technology, Danvers, MA, USA; rabbit polyclonal IgG anti-human NF-κB p65, ref. sc-372, Santa Cruz Biotechnology, Dallas, TX, USA) diluted into blocking buffer (PBS with 5% goat serum). The following day, cells were washed and fluorochrome-conjugated secondary antibodies applied for 1.5 hour at room temperature in the dark (goat polyclonal IgG anti-mouse-AF568, ref. A11004 and goat anti-rabbit-FITC, ref. 65-6111, 1:500, Life Technologies). Cells were washed with PBS three times and coverslipped with Prolong® Gold Antifade Reagent containing DAPI (ref. 8961, Cell Signaling Technology). Images were acquired with an Olympus BX63 microscope (Olympus Life Sciences, Berchem, Belgium) equipped with XM-10 camera (Olympus Life Sciences). For NF-κB p65 translocation assay, coverslips were mounted in Mowiol containing Hoechst nucleus counterstaining. Labeled cells were observed at the 63x magnification on a Leica TCS SP5 confocal microscope (Leica Microsystems CMS GmbH, Wetzlar, Germany) using 488nm laser excitation. Twenty individual cells among those that were in focal field were randomly chosen and the spatial relationship between the NF-κB immunoreactivity and nuclear signal was visually assessed. For each

condition of stimulation, 200 events at least were collected. All of the data were recorded at the same laser and multiplier settings.

Flow cytometry

DPSC markers analysis: Briefly, $3x10^6$ cells were harvested using 0.05% trypsin/EDTA, followed by resuspension in fluorescence-activated cell sorting buffer solution (PBS supplemented with 0.1% BSA, 0.02% azide, 1 mM EDTA, BD Biosciences). The cells were characterized using a human MSC Analysis Kit (Cat. No. 562245, BD Biosciences, Piscataway, NJ, USA) according to the manufacturer's protocol. The kit includes the MSC positive antibody cocktail (FITC mouse Anti-Human CD90, APC Mouse Anti-Human CD73, PerCP-Cy5.5 Mouse Anti-Human CD105) and negative MSC antibody cocktail (PE CD34, PE CD45, PE CD11b, PE CD19 and PE HLA-DR).

Apoptosis analysis: DPSCs were seeded in 6-well plates (Greiner Bio-One GmbH) at a density of 2×10^5 cells/well and allowed to reach full confluence. Then, the samples were incubated in 3mL/well media containing different concentrations of LTA or LPS. After 24h, the cells were trypsinized, centrifuged and resuspended in the medium. The cells were double-stained with PE-conjugated Annexin-V and 7-AAD (Guava Nexin Reagent, Guava Technologies, Hayward, CA, USA). Percentages of live (Annexin-V-/7-AAD-), early apoptotic (Annexin-V+/7-AAD-) or late apoptotic and necrotic cells (Annexin-V+/7-AAD+ and Annexin-V-/7-AAD+) were determined using flow cytometry. All flow cytometry analysis were conducted on a BD FACSCanto II (BD Biosciences, Piscataway), and the data were analyzed by FlowJo LLC software (version 9.7.7, Ashland, OR, USA).

RNA isolation

Total RNA was extracted using High Pure RNA Isolation Kit (Roche Diagnostic GmbH, Mannheim, Germany). RNA concentrations were measured using a spectrophotometer Nanodrop 1000 (Thermo Scientific, Bleiswijk, Netherlands). One µg of total RNA was used as a template to make first-strand complementary DNA by High Capacity cDNA Reverse Transcription Kit according to

manufacturer's instructions, including DNAse treatment (Applied Biosystems, Thermo Fisher Scientific Baltics, Vilnius, Lithuania).

RT-PCR

PCR reactions were performed in a total volume of 20μL containing 1μL of cDNA, 0.5U KAPA2G Fast HotStart DNA polymerase (Kapa Biosystems), 0.2mM dNTP, 0.5μM of each primer, and 10μL KAPA buffer. The PCR conditions were 95°C for 1.5min followed by 35 cycles of 30sec at 95°C, 30sec at 60°C and 1min at 72°C, and final extension at 72°C for 5 min. The PCR amplification products were submitted to electrophoresis on a 1.2% agarose gel in TAE buffer: 40mM Tris, 40mM acetate, 1mM EDTA. Amplicons were visualized after gel exposure to 0.5μg/mL of ethidium bromide and UV transillumination in a GelDoc apparatus (Bio-Rad Laboratories). Primer sequences are listed in Table 1.

qRT-PCR

One μL of cDNA was used to amplify the target genes using specific primer pairs for human TLRs (InVivoGen, San Diego, CA, USA) and Takyon SYBR Green amplification kit (Eurogentec, Liège, Belgium) according to manufacturer's instructions in the Light Cycler 96 Real-Time PCR system (Roche Diagnostics, Mannheim, Germany). Relative gene expression was computed using the 2^{-ddCq} method with human GAPDH as housekeeping gene and compared to the unstimulated condition (LTA or LPS 0 μg/mL). Specific amplification was confirmed by melting curve analysis.

Cell viability assay

DPSCs were plated at a density of $1x10^4$ cells per well and a volume of 100µL in 96-well plate (Greiner Bio-One GmbH). The cells were grown in cell medium culture and allowed to adhere for 12 hours at 37 °C in 5% CO_2. The samples were incubated in media containing different concentrations of LTA or LPS. Cell viability was evaluated by the conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) to a purple color product by cellular mitochondria. 24-hours post-stimulation, cells were washed with sterile PBS and then incubated with 0.5 mg/mL (100µL/well) MTT (VWR, Amresco, OH, USA). Following a 2-hour incubation, the supernatant was removed from the plates and isopropyl alcohol (100µL/well) was added. The absorbance of MTT was measured using a spectrophotometer (Multiskan Ascent 354 microplate reader, Labsystems, Vantaa, Finland) at 540nm.

Adhesion assay

Ninety six-well plates (Greiner bio-one GmbH) were coated with 50 µL type I collagen (40mg/L in 1xPBS dilution) (Collagen rat tail, Roche Diagnostics GmbH, Mannheim, Germany) overnight at 4°C. The wells were washed twice with 1xPBS to remove unbound collagen and blocked with 1% BSA for 1 hour at 37 °C in 5% CO_2. $1x10^4$ cells were added to each well in the presence of different concentrations of LTA or LPS. Cells were incubated for 90 min at 37 °C in 5% CO_2. The wells were then washed three times with 1xPBS. The cells were fixed with 200µL 4% paraformaldehyde per well at room temperature for 15 min and washed three times with 1xPBS. The cells were stained with 100µL/well 0.1% crystal violet for 10 min at room temperature. The wells were washed three times with ddH_2O prior to the addition of 100µL of 10% acetic acid to each well. The optical density was measured using a spectrophotometer (Multiskan Ascent 354 microplate reader, Labsystems) at 595 nm.

Transwell migration assay

Cell culture inserts with 8µm pores (ThinCert™, Greiner bio-one GmbH) in a 24-well plate (Greiner bio-one GmbH) were used in this experiment. DPSCs ($1x10^5$ cells per insert) suspended within 400µL of standard medium were seeded onto the membrane of the upper chamber. The cells were allowed to adhere to the filters for 12 hours at 37 °C in 5% CO_2. After cell adherence, the lower chamber was filled

with culture medium containing different concentrations of LTA or LPS. 24 hours post-stimulation, non-migrated cells on the upper side of the membrane were removed with a cotton swab. The number of migrated DPSCs on the lower side of the membrane was determined via Cell Titer 96® AQ_{ueose} One Solution Cell Proliferation Assay (MTS, Promega GmbH, Mannheim, Germany) according to manufacturer's instructions. For this purpose, the medium of the lower chamber of the transwell assay was replaced by standard medium containing 16.7% MTS solution and transwell assays were incubated for 1 h under standard conditions. Finally, medium was transferred to an optical 96-well plate (Greiner bio-one GmbH) and absorbance was measured at 490 nm in a microplate reader (Multiskan Ascent 354 microplate reader, Labsystems).

Wound healing assay

In total, $2x10^5$ DPSCs were seeded into 6-well plate and allowed to reach full confluence. A wound was inflicted by making a scratch through the cell monolayer with a 200 µL pipette tip. Three parallel horizontal lines and three parallel vertical lines were scratched on the bottom of each well (Greiner Bio-One GmbH) to create 9 intersections. After wounding, cells were washed with PBS to remove debris and then incubated with cell culture medium in the presence of different concentrations of LTA or LPS for 24h. Microphotographs of the scratch were taken at 0, 6, 12, 18 and 24 hours post-wounding. The cultured cells were observed with a phase-contrast microscope (Olympus, Tokyo, Japan). Wound closure rate was determined at each intersection by image analysis using ImageJ (National Institute of Health, Bethesda, MD, USA).

Enzyme-linked immunosorbent assay

In total, $2x10^5$ DPSCs per well were seeded in 6-well plate and allowed to reach full confluence. Then, the samples were incubated in 3mL/well media containing different concentrations of LTA or LPS. Twenty-four hours post-stimulation, the supernatants were collected and stored at -80 °C until assay. Levels of IL-1β (Affymetrix eBioscience, Thermo Fisher Scientific), IL-6, IL-8 (CX-CL8), IL-10, IL-12, MCP-1, TNF-α and IFN-γ in the culture supernatants were analyzed using commercially available ELISA kits according to the manufacturer's protocol (Novex® Life technologies, Invitrogen). Briefly, 96-well plates (ThermoFisher Scientific) were coated overnight at 4°C with capture antibody diluted

1000x in PBS. Plates were washed once with wash buffer and blocked for 2 hours with Reagent Diluent. The plates were then washed 4 times again, incubated with detection antibody and the manufacturer's standards as well as the samples were added to the plates for 2 hours. After washing 3-5 times, streptavidin-horseradish peroxidase was added for 45 minutes. The plates were washed 5-7 times and incubated with substrate solution (TMB) for 30 minutes. At the end, a stop solution was added and optical density was measured using a microplate reader (Multiskan Ascent 354 microplate reader, Labsystems) at 450nm.

Differentiation assays

Osteogenic differentiation

DPSCs were seeded in 6-well plate (Greiner Bio-One GmbH) at a density of 2×10^4 cells/well and incubated with respective differentiation media. To push toward osteogenic differentiation, DPSCs were cultured in basal medium supplemented with 0.1µM dexamethasone, 10mM β-glycerophosphate (Sigma-Aldrich, St-Louis) and 50µg/ml ascorbic acid. DPSCs were differentiated up to 7, 14, 21 and 28 days replacing the medium every 3 days. The first day and every three days, different concentrations of LTA or LPS 1µg/mL were added to the differentiation medium. At the indicated times, the cells were washed 3 times with 1xPBS and fixed with 4% paraformaldehyde for 15 minutes at room temperature. To assess osteogenic induction, Alizarin Red S assay has been used to evaluate the amount of calcium deposits in cell culture. The fixative was removed and the cells were washed 3 times with ddH_2O prior to staining with 40mM Alizarin Red S (Sigma–Aldrich) 1mL/well, 30 minutes with gentle shaking at room temperature. Unbound dye was removed by several washes with ddH_2O. The samples were dissolved in 10% acetic acid, were transferred to 1.5mL microcentrifuge tube and were heated at 85 °C for 10 minutes. The samples were centrifuged at 16.000g for 15 minutes and then transferred to a new tube. 10% ammonium hydroxide was added to neutralize the acid and obtain a pH between 4.1 and 4.5. The samples were transferred to 96-well plate (Greiner Bio-One GmbH) in triplicate. Dye was quantified spectroscopically at 405 nm extrapolating the concentration using a standard curve.

Adipogenic differentiation

DPSCs were seeded in 6-well plate (Greiner Bio-One GmbH) at a density of 2×10^4 cells/well. To push toward adipogenic differentiation, DPSCs were cultured in basal medium supplemented with 1µM dexamethasone, 0.5mM IBMX (3-isobutyl-1-methylxantine, Sigma-Aldrich) and 100µM indomethacin and 10µg/mL insulin. DPSCs were differentiated up to 21 days replacing the medium every 3 days. At the indicated times, the cells were washed 3 times with 1xPBS and fixed with 4% paraformaldehyde for 15 minutes at room temperature. To assess adipogenic induction, Oil Red O and Soudan black assays have been used to evaluate the amount of lipid deposit in cell culture. The fixative was removed and the cells were washed 3 times with ddH_2O prior to staining with Oil Red O and Soudan black.

Chondrogenic differentiation

DPSCs were seeded in 6-well plate (Greiner Bio-One GmbH) at a density of 2×10^4 cells/well. To push toward chondrogenic differentiation, DPSCs were cultured in basal medium supplemented with 0.1µM dexamethasone, 100µg/mL sodium pyruvate, 10ng/mL TGFß3 (Sigma-Aldrich), 50µg/mL acid ascorbic and 10µg/mL insulin. DPSCs were differentiated up to 21 days replacing the medium every 3 days. At the indicated times, the cells were washed 3 times with 1xPBS and fixed with 4% paraformaldehyde for 15 minutes at room temperature. The extent of chondrogenic differentiation was assessed by staining the cells with 0.1% Safranin-O staining assay. The fixative was removed and the cells were washed 3 times with ddH_2O prior to staining with 0.1% Safranin-O.

Statistical analysis

Results were expressed as mean values ± Standard Error of Mean (SEM) obtained from at least three independent experiments. Each assay was repeated three times on independent batches of DPSCs (from different donors). The data collected were statistically analyzed using one-way or two-way ANOVA. The level of significance was set at $p<0.05$. Tukey post-hoc multiple comparison tests were carried out to determine the differences between groups. The statistical analyses were performed using GraphPad Prism version 7 (GraphPad Software Inc., La Jolla, CA, USA).

Results

Characterization of adult DPSCs

DPSCs had typical morphology of fibroblast-like cells and expressed high levels of intermediate filaments vimentin and CD44 (Fig. 1A). Common mesenchymal stem cells markers (CD73, CD90 and CD105) were consistently positive, whereas hematopoietic markers (CD34, CD45, CD11b, CD19 and HLA-DR) were negative in all samples tested (Fig. 1B). RT-PCR analysis revealed the expression of stemness-related transcription factors as Oct3/4, Sox2, Nanog and Nestin in DPSCs, which were absent in fully differentiated keratinocytes used as negative control (Fig. 1C). The multipotency of DPSCs was evaluated through incubation with osteogenic, chondrogenic or adipogenic differentiation media. Upon three weeks of differentiation, differentiated DPSCs showed the abilities to secrete either a calcified matrix or high amount of proteoglycans, or to accumulate intracellular lipids (Fig. 1D)

DPSCs express TLR2 and activate NF-κB pathway upon LTA stimulation

RT-PCR analysis revealed TLR1, TLR2 & TLR6 expression in DPSCs (Fig. 2A). LTA treatment induced nuclear NF-κB p65 translocation for 0.1 and 1 µg/mL but not for the highest dose. The amount of translocated NF-κB p65 in LTA-stimulated DPSCs was however significantly lower than LPS-stimulated DPSCs ($P \leq 0.001$) (Fig. 2B). To assess whether LTA was able to stimulate the expression of its own receptor in DPSCs, TLR2 mRNA expression and associated receptors was measured following 6 hours or 24 hours of LTA exposure. qRT-PCR data revealed that after treatment with different concentrations of LTA, TLR1, -2 or -6 expressions were not modulated at any time (Fig. 2C). However, LPS-stimulated DPSCs significantly upregulated TLR1 ($P \leq 0.05$), TLR2 ($P \leq 0.001$) and TLR6 ($P \leq 0.001$) (Fig. 2C).

LTA stimulates DPSCs proliferation and does not induce cell death

Incubation for 24 hours with LTA induced the proliferation of DPSCs, with the highest proliferation recorded for LTA 1µg/mL ($P \leq 0.001$) and 10 µg/mL ($P \leq 0.01$). Similarly, LPS 1µg/mL stimulated the proliferation of DPSCs ($P \leq 0.05$) (Fig. 3A). Intriguingly, this effect was lost for both LTA and LPS at 48-hour, 72-hour and 10-days of stimulation (data not shown). As the number of cells per well is also function of cell death rate, the proportion of viable cells or of cells entering in early apoptosis, late

apoptosis or necrosis was evaluated. Whatever the dose or timing of incubation with LTA or LPS, no changes in proportion of living or apoptotic cells were noticed (Fig. 3B).

LTA influences cell-to-matrix adhesion and accelerates horizontal migration

Treatment with LTA 0.1μg/mL significantly increased adherent cell numbers compared with unstimulated group ($P \leq 0.01$). However, treatment with LTA 1μg/mL, 10μg/mL and LPS 1μg/mL significantly ($P \leq 0.05$) decreased adherent cell numbers compared with the unstimulated group (Fig.4A). A two-chamber transwell system was also used to explore the effect of LTA and LPS on the vertical migratory abilities of DPSCs. The results revealed no difference in the migratory ability upon LTA or LPS stimulation (Fig.4B). The scratch assay was used to investigate the effect of LTA on the horizontal migratory abilities of DPSCs. Treatment with LTA 1μg/mL and LPS 1μg/mL enhanced significantly DPSCs migration toward the scratch area at 18 hours ($P \leq 0.01$) and 24 hours ($P \leq 0.001$) (Fig. 4C). Although the cells started to migrate towards the scratch area 12 hours after it was inflicted in all conditions, the space width closure in LTA 1 μg/mL group was accelerated significantly more than in unstimulated groups.

LTA induces cytokine production in DPSCs

LTA did not induce the production of MCP-1 by DPSCs although LPS did (Fig. 5A). LPS 1 μg/mL, used as a potent inducer of chemokine production, also induced the secretion of IL-8, having no effect on IL-6 secretion. The level of expression of other cytokines such as IL-1β, IL-10, IL-12, TNF-α and IFN-γ was unchanged between LTA-stimulated cells and unstimulated cells (data not shown). However, 24 hours after incubation with different concentrations of LTA, the production of IL-6 ($P \leq 0.0001$) and IL-8 increased in dose-dependent manner (Fig. 5B-C). This effect was similarly observed at 48- and 72-hours of LTA incubation (data not shown). To prove that IL-8 secretion was consequent to specific TLR2 binding and signalling, LTA-stimulated cells were co-incubated with CCPT22, a known TLR2/1 antagonist. CU-CPT22, at a non-toxic concentration of 10μM, significantly inhibited LTA-induced IL-8 upregulation (Fig. 5D)

LTA does not modulate the osteogenic differentiation

DPSCs cultured in a specifically formulated medium supporting the differentiation into cells of the osteogenic lineage starts to mineralize the extracellular medium between the 14th and 21st day compared to cells earlier in the differentiation process ($P \leq 0.001$). Cells were co-exposed to this differentiation medium and to increasing concentrations of LTA (Fig. 6, left panel) or LPS 1 µg/mL (Fig. 6, right panel). There were no significant differences in the amount of calcium deposits at day 21 or 28 of osteogenic differentiation between unstimulated and stimulated cells.

Discussion

In the initial steps of dental caries, cariogenic bacteria contained in biofilms produce organic acids lowering pH to values between 5.0 and 5.5, to locally induce the slow demineralization of adjacent enamel. If the enamel barrier becomes disrupted and if the carious process remains untreated, bacteria will progress through the dentine tubules, up to the pulp (Love & Jenkinson 2002). *Streptococcus* spp, *Actinomyces* spp, *Lactobacillus* spp and *Bifidobacterium* spp largely dominate the carious microflora at this early stage and all belong to the Gram-positive classification. *Enterococcus faecalis*, another Gram-positive bacterium expression LTA, easily survives in dentinal tubuli even after careful disinfection or antibioprophylaxis, and is one of the most common factor associated with post-treatment endodontic disease. In this context, the present study sought to mimic, in a laboratory setting, a clinical-relevant condition where DPSCs would come into direct contact with LTA, a Gram-positive cell surface major component. This is the first study that addresses the effect of clinical relevant concentrations of LTA on DPSCs properties. The results demonstrated that human DPSCs were able to sense extracellular LTA likely through TLR2 basal expression, and consequently able to activate, for a minority of the cells, the NF-κB pathway as a result of receptor ligation. Hence, LTA promoted the proliferation, the horizontal migration and inflammatory cytokine production of DPSCs, without interfering with the osteogenic differentiation abilities of DPSCs. Potentially-activated intracellular pathways driving the observed biological effects were also explored but we were not able to detect any phosphorylation of Akt, p38 or ERK signaling partners (data not shown).

DPSCs expressed *in vitro* functional TLRs involved in the recognition of LTA or di-/tri-acyl lipopeptides (TLR1, -2 and -6). LTA is widely considered as a relevant TLR2 agonist's ligand. Although Gram-positive LTA is a preferential TLR2 ligand, several lipoproteins from Gram-negative bacteria have also been identified as TLR2 ligands, due to N-terminal sequences containing diacylated or triacylated cysteines (Oliveira-Nascimento *et al.* 2012). Upon LTA stimulation, translocation of NF-κB p65 subunit to the nucleus was detected in a tiny fraction of cells, attesting for a functional receptor signaling, but to a well lesser extent than with LPS stimulation. Whether LTA would regulate the expression of its own receptors on DPSCs, as already described for LPS and its receptor TLR4 (Liu *et al.* 2014) was also tested. The results revealed that LTA did not upregulate the gene expression of TLR1, -2 or -6 in DPSCs, contrary to LPS that was a potent inducer. This suggests that, under infectious conditions, TLR2 upregulation in DPSCs is mostly mediated by exposure to LPS from Gram-negative bacteria than to LTA from Gram-positive bacteria. Unlike pulp stem cells, odontoblasts increase the expression of TLR2 upon LTA stimulation (Durand *et al.* 2006).

One of the limitations of the study is the use of a commercially-available LTA purified from *Staphylococcus aureus*, and not from *Streptococcus mutans*, the major cariogenic Gram-positive bacteria that would have more closely mimicked the pathological carious condition. LTAs are present on most, if not all, Gram-positive bacteria including Streptococcus spp (Hogg *et al.* 1997, Schneewind & Missiakas 2017). According to their origins and structures, LTAs exhibit differential immunostimulatory potentials, with *Staphylococcus aureus*-derived LTA acting as one of the most potent immunostimulator on peripheral blood mononuclear cells or macrophages (Hermann *et al.* 2002, Hong *et al.* 2014). The experimental design consisted of exposing the cells to increasing concentrations of LTA or to LPS in a separate well serving as a positive control of the read-out. LTA aliquots had an undetectable LPS contamination; so that LPS traces were considered as not responsible for the observed biological effects. Overall, LTA stimulation induced a more moderated response compared to LPS in regards to NF-κB activation, TLRs upregulation, cell proliferation and migration, and finally cytokine production. While LTA and LPS share many pathogenic properties, there are findings that do not clearly support the role of LTA as a major virulence factor; because quantities of LTA (µg to mg/mL) necessary

to induce the secretion of proinflammatory mediators are many times greater than those of LPS (ng/mL). On a weight-for-weight basis, LTA seems less immunogenic than LPS. Accordingly, this report supports the hypothesis that LTA is a weaker factor of virulence in comparison to LPS (Parolia *et al.* 2014), although DPCSs are able to sense Gram-positive derived LTA and upon ligation, they mainly trigger the secretion of such IL-8 and IL-6.

LTA-stimulated DPSCs reached a higher proliferation level compared to unstimulated cells, this result was linked to a real increase in cell number upon TLR2 ligation and not a decreased number of cells undergoing apoptosis or necrosis in culture conditions. The data contrasts with previous information demonstrating a high apoptosis rate in dental pulp cells exposed to *Streptococcus mutans*-LTA (Wang *et al.* 2001). Of note, the concentrations of LTA used in the present study were much lower than in Wang *et al.* (2001) and probably reflect more the clinical reality (Wang *et al.* 2001). Intriguingly, adhesion of DPSCs to collagen matrix was affected in a differential way according to LTA concentrations; with the lowest dose (0.1 µg/mL) the adherence was increased and the highest doses (1 and 10 µg/mL) slightly decreased. Whereas vertical migration through a Boyden chamber was unaffected at any concentrations investigated, the scratch assay clearly demonstrated that DPSCs stimulated with LTA 1µg/mL enhanced their mobility. Also, LPS-stimulated DPCSs showed enhanced horizontal migration compared with control, this confirmed previous data on the effect of LPS (Li *et al.* 2014). A short-term wound-healing assay (<24h), as performed, will most likely assess migration and to a lesser extent proliferation (Kramer *et al.* 2013). Nevertheless, it cannot be ruled out that increased proliferation and/or the production of cytokines or chemokines following TLR2 activation contributes to the observed migratory phenotype. A well-recognized consequence of TLR2 ligation is the upregulation of cytokine synthesis in various cell types (Cook *et al.* 2004), including dental pulp cells (Durand *et al.* 2006, Staquet *et al.* 2008). Noteworthy, the profile or levels of secreted cytokines differed slightly between LPS- and LTA-stimulated DPSCs, as suggested in other cell types (Finney *et al.* 2012). In this study, it is shown that LTA did not influence the speed of osteogenic/odontoblastic differentiation when DPSCs were exposed to the appropriate medium. Unexpectedly, although the experiment was repeated three times, it was not provided to replicate the findings that LPS promotes the odontoblastic differentiation of DPSCs (He *et al.* 2015). This contradictory data may result from slight differences in

the differentiation protocol, cell confluency at the start of induction or the serotype of E. coli used for LPS isolation. Supporting a variable response towards LPS stimulation, the osteogenic differentiation of dental pulp cell lines (Nomiyama *et al.* 2007), periodental ligament stem cells (Albiero *et al.* 2017) or dental follicle cells (Morsczeck *et al.* 2012) is either inhibited or unaffected accordingly.

Conclusion

This paper describes an (immuno-)stimulatory effect of LTA on human DPSCs. It demonstrates that pulp stem cells do sense the Gram-positive cell wall component and can intiate NF-κB-dependent signaling leading to modest proliferation, enhanced migration and significant cytokine secretion. Despite the use of LTA derived from a non-oral bacteria (*S. aureus*), the present study reports that DPSCs respond to the presence of oral Gram-positive bacteria, such as *Streptococcus mutans*, reaching the pulp cavity during a carious process (Liu *et al.* 2014). Such findings help to understand how DPSCs behave following contact with oral cariogenic bacteria and can serve as a catalyst for designing novel strategies to boost regenerative properties of DPSCs despite an inflammatory or infectious environment.

Acknowledgements

The authors wish to thank Professors Patricia Renard, Astrid Vanden Abbeele and Yves Poumay for providing respectively NF-κB p65 antibody, mesenchymal stem cell markers and RNA library from human keratinocytes. We warmly thank Prof. Jacques Gilloteaux for English proof-reading and Pascale Deblandre, Noël Ninanne and Benoit Balau for their technical assistance. This research used the microscope facilities of the "Plateforme Technologique Morphologie–Imagerie" of UNamur.

Conflict of Interest

The authors have stated explicitly that there are no conflicts of interest in connection with this article.

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Figure Legends

Figure 1 Phenotypic characterization of DPSCs. (A) Double immunofluorescent labeling showing cytoplasmic expression of vimentin (green) and CD44 (red) counterstained with DAPI. All cells in the field-of-view have a spindle-shaped morphology and co-express CD44 and vimentin. Scale bars = 20 µm (B) Flow cytometric analysis confirms the expression of mesenchymal stem cell markers CD73, CD90, CD105 by DPSCs, without expression of CD11b, CD19, CD34, CD45 and HLA-DR. The percentage of positive cells for each marker is shown. The unlabeled cells and the IgG isotype control are represented as red and blue curves respectively. (C) The expression of stemness-related transcription factors Oct3/4, Sox2, Nanog and Nestin is detected in DPSCs (d) and absent in differentiated keratinocytes (k). (D) Upon 3 weeks of exposure to respective differentiation medium, DPSCs are able to commit towards osteogenic (Alizarin Red), chondrogenic (Safranin O) or adipogenic (Oil Red O and Soudan Black) lineages.

Figure 2 Expression of TLR1, -2 and -6 and NFKB p65 activation upon LTA stimulation. (A) TLR1, TLR2 and TLR6 mRNAs are expressed under basal conditions by DPSCs. (B) Unstimulated DPSCs show a diffuse cytoplasmic NKFB p65 labeling while significant nuclear translocation is observed upon LPS exposure. In DPSCs exposed to LTA 0.1 and 1 µg/mL, few translocations of p65 to the nucleus are observed. Scale bars = 25 µm (left panel) or 50 µm (central and right panels). Quantification of p65-immunoreactive nuclei shows that LPS 1µg/mL and LTA 0.1 µg/mL significantly increase the translocation when compared to unstimulated cells. (C) LTA does not modulate the gene expression of TLR1, TLR2 or TLR6 after 6 hours or 24 hours of exposure. LPS, used as positive control, significantly upregulates TLR1, TLR2 and TLR6 mRNA expression. Data are presented as mean ± SEM. *$p \leq 0.05$, **$p \leq 0.01$, ****$p \leq 0.0001$ LPS compared to unstimulated and LTA; #$p \leq 0.05$, ##$p \leq 0.01$ LTA compared to unstimulated cells (0 µg/mL).

Figure 3 Viability assessment of DPSCs and cell death upon LTA stimulation. (A) 24 hours of exposure to LTA 1 and 10 µg/mL significantly increases the number of living cells in the wells. Similarly, LPS 1 µg/mL for 24 hours stimulates the cell viability. Cell viability rate (%) is expressed as a percentage of O.D. of MTT reaction related to the unstimulated condition. (B) Proportion of living or dead cells (%) is quantified by FACS expression of markers Annexin-V and/or 7-AAD.

Results are expressed as a percentage of total labeled cells. Annexin-V negative and 7-AAD negative cells are considered as living cells; Annexin-V positive and 7-AAD negative cells are considered as early apoptotic cells; 7-AAD positive cells are considered as late apoptotic or necrotic cells. 24 hours of exposure to LTA does not modify the proportion of living, early apoptotic or late apoptotic cells. LPS 1 µg/mL for 24 hours does not induce significant cell death compared to unstimulated condition. Data are presented as mean ± SEM. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 LPS-stimulated or LTA-stimulated DPSCs compared to unstimulated cells (0 µg/mL). Each graph illustrates one representative experiment out of three independent experiments.

Figure 4 Effect of LTA on adhesion and migration of DPSCs. (A) The adhesion ability of DPSCs exposed for 24 hours to various concentrations of LTA and LPS 1µg/ml. Treatment with LTA 0.1µg/mL increased cell adherence to collagen matrix compared with unstimulated group. However, treatment with LTA 1µg/mL, 10µg/mL and LPS 1µg/mL significantly decreased adherent cell numbers compared with unstimulated group. (B) Vertical migration was evaluated using a two-chamber Transwell system with pore diameter of 8µm. There was no statistical difference between the different conditions stimulated with LTA or LPS. (C) Horizontal migration was evaluated using a wound-healing assay onto confluent monolayers. Relative cell migration rate in each group was calculated based on the closure of the initial gap and expressed in % of wound closing. The treatment with LTA 1µg/mL, and LPS 1µg/mL significantly promoted cell migration when compared with unstimulated group at 18h and 24h. Data are presented as mean ± SEM. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 LPS-stimulated or LTA-stimulated DPSCs compared to unstimulated cells (0 µg/mL). n.a. not assessed. Each graph illustrates one representative experiment out of three independent experiments.

Figure 5 Effect of LTA on cytokine release. Cytokine content is assessed by ELISA in the supernatant of in vitro culture of DPSCs stimulated for 24h with various concentrations of LTA or LPS 1 µg/mL. (A) The release of MCP-1 is not modified when DPSCs are exposed to various concentrations of LTA. However, the production of MCP-1 is highly upregulated upon LPS stimulation in comparison with the unstimulated group. (B) The production of IL-6 increases after LTA exposure in a dose-dependent manner. There is a slight increase production of IL-6 after LPS stimulation but dos not reach statistical significance. (C) The production of IL-8 increases after LTA exposure in a dose-dependent manner.

There is a greater increase of IL-8 production after LPS stimulation. (D) CU-CPT-22, a specific TLR2 antagonist, significantly inhibits LTA-induced IL-8 upregulation. Data are presented as mean ± SEM. *p ≤ 0.05, ***p ≤ 0.001, ****p ≤ 0.0001 LPS-stimulated or LTA-stimulated DPSCs compared to unstimulated cells (0 µg/mL); #p ≤ 0.05, ##p ≤ 0. 01, for comparisons between CU-CPT-22 and DMSO-treated DPSCs. Each graph illustrates one representative experiment out of three independent experiments.

Figure 6 Influence of LTA on the osteogenic potential of DPSCs. The osteogenic differentiation of DPSCs exposed to different concentrations of LTA or LPS 1µg/mL is assessed by the quantification of extracellular calcium deposits. Once osteogenic differentiation medium is added to DPSCs, the cells progressively mineralize the extracellular matrix with a drastic induction of calcium deposition between the 14th and 21th day of differentiation. Quantitative analysis shows that LTA, at any dose, does not change the amount of calcium deposits over time. Similarly, there is no statistical difference when DPSCs are exposed to LPS 1µg/mL during the differentiation process. Data are presented as mean ± SEM. Each graph illustrates one representative experiment out of three independent experiments.

Supplementary Figures

Supplementary Figure 1 HEK-Blue™-hTLR2 reporter cells expressing human TLR2 and secreted alkaline alkaline phosphatase (SEAP) were used to validate S. aureus LTA as hTLR2 agonist. The SEAP reporter gene is placed under the control of the IFN-β minimal promoter fused to five NF-κB and AP-1-binding sites. Upon agonist stimulation, the intracellular receptor domains recruit and activate NF-κB and AP-1 which subsequently induce the production of SEAP, whose activity can be detected using HEK-Blue detection culture medium at an optical density (O.D.) of 630nm. 50.000 HEK-hTLR2 cells were seeded in 24-well and stimulated with LTA (0.0 ; 0.1 ; 1.0 ; 10 µg/mL) or LPS (1 µg/mL) for 24 hours. Upon LTA stimulation, there is a dose-dependent activation of TLR2 signaling in HEK-Blue™-hTLR2 reporter cells.

Table 1 Primer sequences for RT-PCR and qRT-PCR.

Primer	Sequence forward (5'-3')	Sequence reverse (5'-3')	Amplicon size (bp)
GAPDH	CTGGGCTACACTGAGCACC	AAGTGGTCGTTGAGGGCAATG	101
Oct3/4	GGAGAGCAACTCCGATGG	TTGATGTCCTGGGACTCCTC	110
Nanog	TTTGTGGGCCTGAAGAAAACT	AGGGCTGTCCTGAATAAGCAG	116
Nestin	CTGCTACCCTTGAGACACCTG	GGGCTCTGATCTCTGCATCTAC	141
Sox2	GCCGAGTGGAAACTTTTGTCG	GGCAGCGTGTACTTATCCTTCT	155
TLR1	TATCTGAGCTTTGGACTTCTGACAT	TCAAGGAAGTGCCAGAATAATCA	728
TLR2	CCCTGGGCAGTCTTGAACATT	GCCTCCGGATTGTTAAGCTTT	656
TLR6	CCTACCGCTGAAAACCAAAGTCTTA	CAAGGTGAAGGGTTTTTGCATTC	457

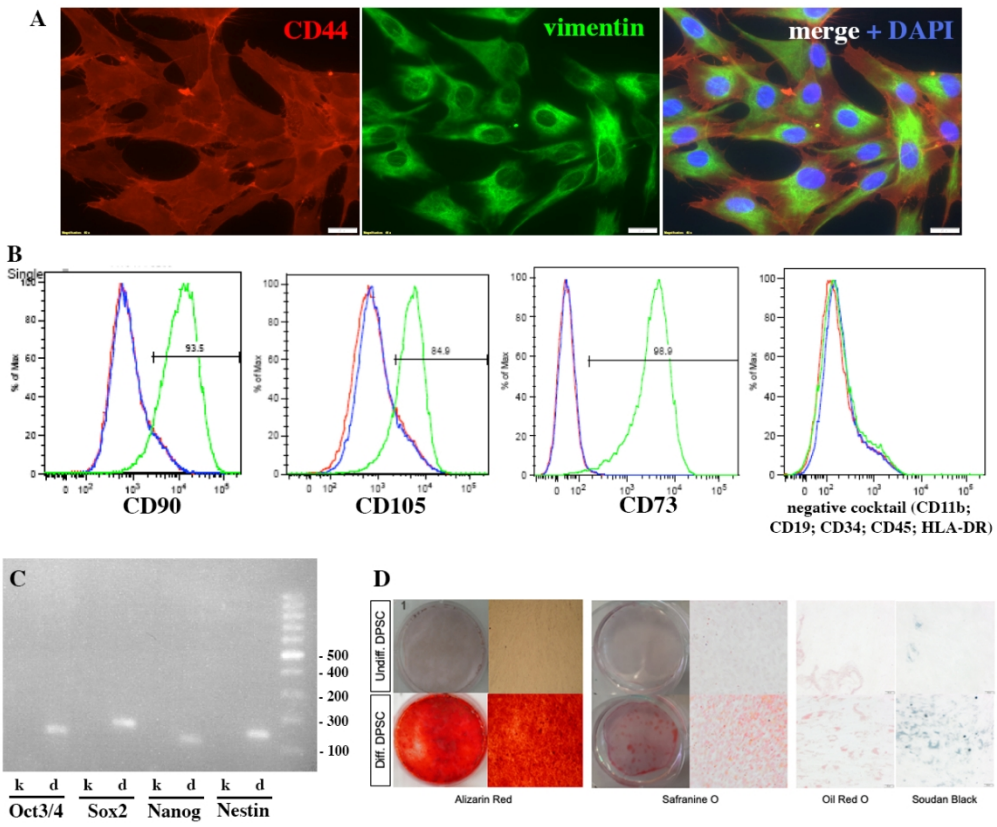
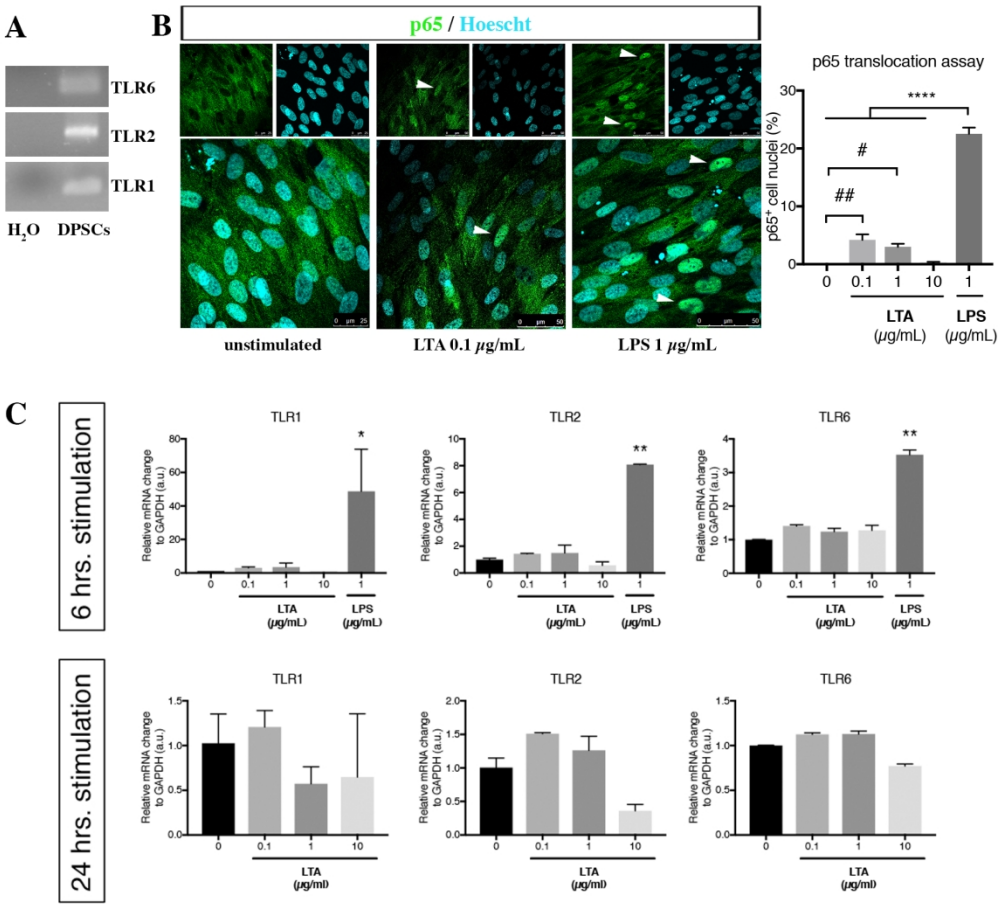
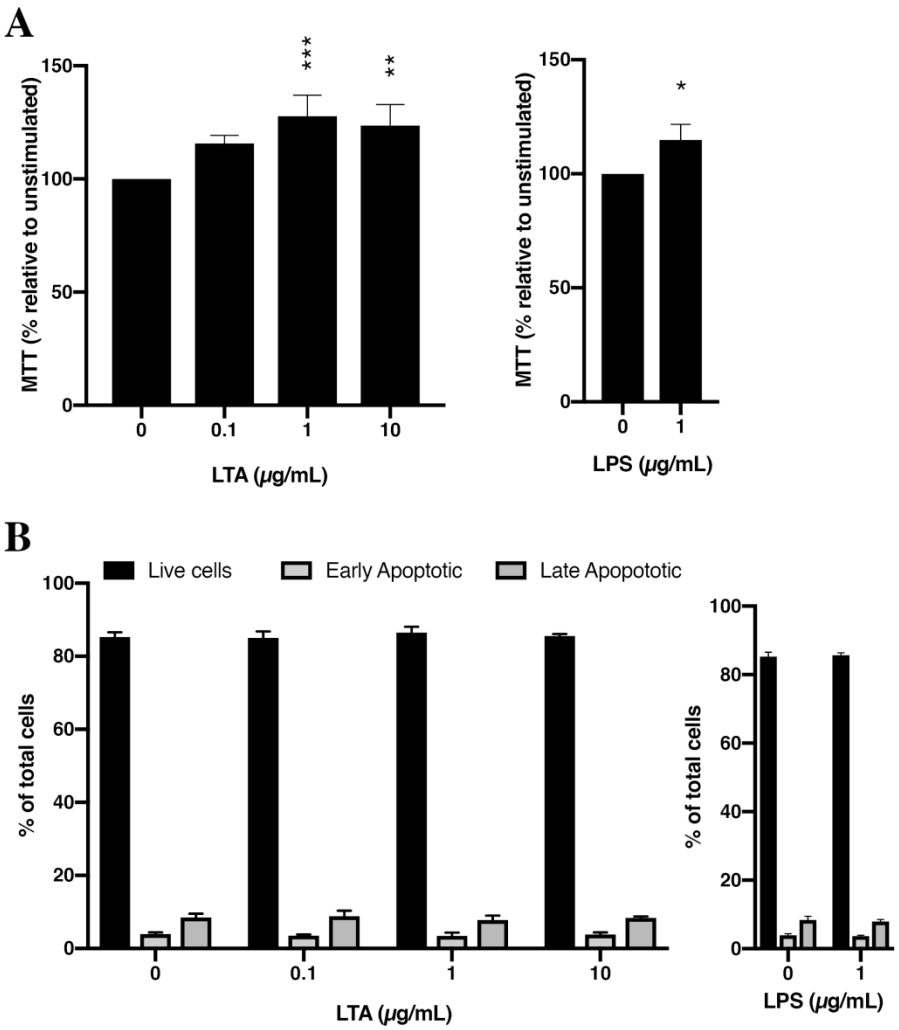
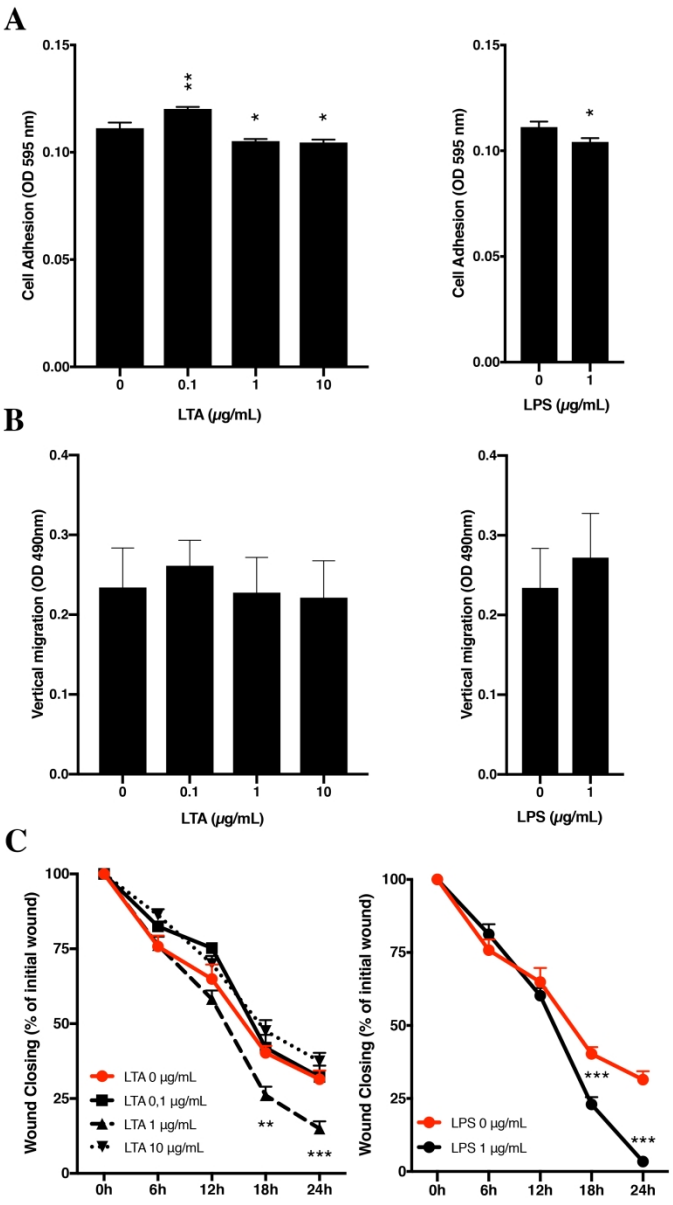

A
CD44
vimentin
merge + DAPI
B
93.5
84.9
98.9
CD90
CD105
CD73
negative cocktail (CD11b; CD19; CD34; CD45; HLA-DR)
C
500
400
200
300
100
k d k d k d k d
Oct3/4 Sox2 Nanog Nestin
D
Undiff. DPSC
Diff. DPSC
Alizarin Red
Safranine O
Oil Red O
Soudan Black

Figure 1


A
TLR6
TLR2
TLR1
H2O
DPSCs
B
p65 / Hoescht
unstimulated
LTA 0.1 μg/mL
LPS 1 μg/mL
p65 translocation assay
p65+ cell nuclei (%)
****
#
##
0
0.1
1
10
1
LTA (μg/mL)
LPS (μg/mL)
C
6 hrs. stimulation
24 hrs. stimulation
TLR1
TLR2
TLR6
Relative mRNA change to GAPDH (a.u.)
*
**
**
LTA (μg/mL)
LPS (μg/mL)


A
MTT (% relative to unstimulated)
150
100
50
0
***
**
0
0.1
1
10
LTA (μg/mL)
MTT (% relative to unstimulated)
150
100
50
0
*
0
1
LPS (μg/mL)
B
Live cells
Early Apoptotic
Late Apopototic
% of total cells
100
80
60
40
20
0
0
0.1
1
10
LTA (μg/mL)
% of total cells
100
80
60
40
20
0
0
1
LPS (μg/mL)


A
Cell Adhesion (OD 595 nm)
0.15
0.10
0.05
0.00
0
0.1
1
10
LTA (μg/mL)
Cell Adhesion (OD 595 nm)
0
1
LPS (μg/mL)
B
Vertical migration (OD 490nm)
0.4
0.3
0.2
0.1
0.0
LTA (μg/mL)
Vertical migration (OD 490nm)
LPS (μg/mL)
C
Wound Closing (% of initial wound)
100
75
50
25
0
0h
6h
12h
18h
24h
LTA 0 μg/mL
LTA 0,1 μg/mL
LTA 1 μg/mL
LTA 10 μg/mL
LPS 0 μg/mL
LPS 1 μg/mL

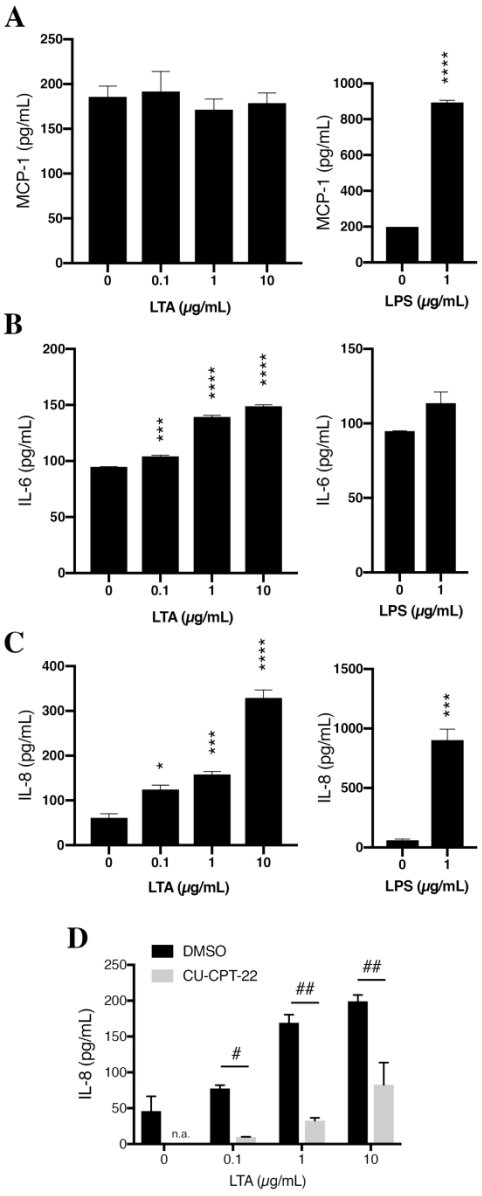
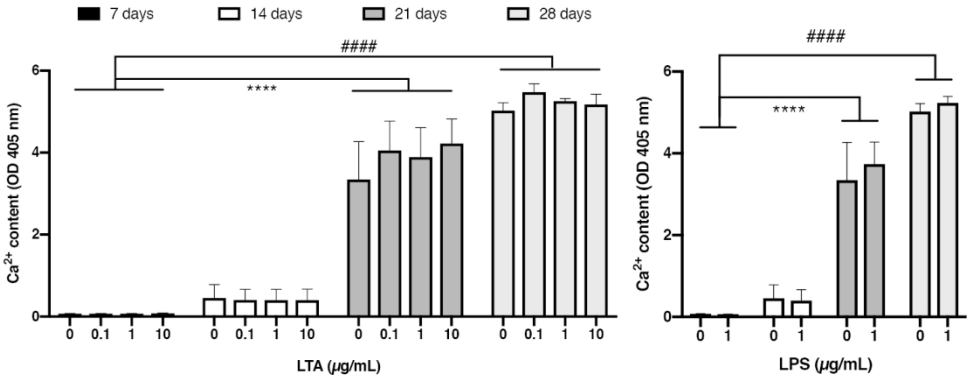
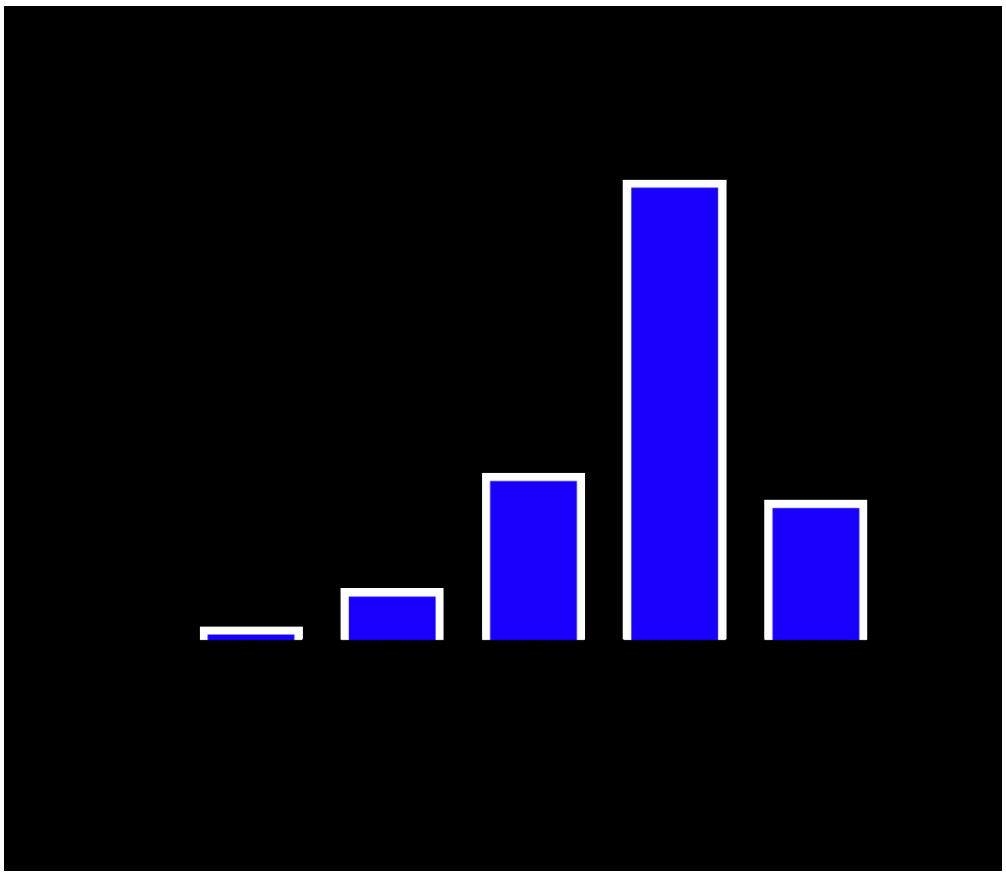

A
MCP-1 (pg/mL)
LTA (μg/mL)
LPS (μg/mL)
B
IL-6 (pg/mL)
LTA (μg/mL)
LPS (μg/mL)
C
IL-8 (pg/mL)
LTA (μg/mL)
LPS (μg/mL)
D
DMSO
CU-CPT-22
IL-8 (pg/mL)
n.a.
LTA (μg/mL)

Figure 5


7 days
14 days
21 days
28 days
####
****
Ca2+ content (OD 405 nm)
0 0.1 1 10 0 0.1 1 10 0 0.1 1 10 0 0.1 1 10
LTA (μg/mL)
####
****
0 1 0 1 0 1 0 1
LPS (μg/mL)



Supplementary Figure 1

100x87mm (300 x 300 DPI)