

TLR signaling can modify the mineralization of tooth germ

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Abstract:

Objective: The aim of this work is to investigate the possible role of Toll-like receptor 4 [TLR4] during the development of mouse tooth germ. TLR4 is well known to inhibit mineralization and cause inflammation in mature odontoblasts and dental pulp cells. However, unlike these pathological functions of TLR4, little is known about the developmental function[s] of TLR4 during tooth development.

Material and methods: TLR4 expression was studied via Western blot in developing lower mouse incisors from E13.5 to E18.5. To generate functional data about the effects of TLR4, a specific agonist [LPS] was applied to the medium of *in vitro* tooth germ cultures, followed by Western blot, histochemical staining, ELISA assay, *in situ* hybridization and RT-qPCR.

Results: Increased accumulation of biotin-labeled LPS was detected in the enamel organ and in preodontoblasts. LPS treatment induced degradation of the inhibitor molecule [IkB] of the NF- κ B signaling pathway. However, no morphological alterations were detected in cultured tissue after LPS addition at the applied dosage. Activation of TLR4 inhibited the mineralization of enamel and dentin, as demonstrated by alizarin red staining and as decreased levels of collagen type X. mRNA expression of ameloblastin was elevated after LPS administration.

Conclusion: These results demonstrate that TLR4 may decrease the mineralization of hard tissues of the tooth germ and may trigger the maturation of ameloblasts; it can give valuable information to understand better congenital tooth abnormalities.

Key words: LPS, enamel organ, development, incisor

Introduction

Tooth germ tissues originate from epithelial and neural crest cells, and their interactions are regulated by sequential and reciprocal interactions [1]. Interactions are controlled by several signaling pathways that are crucial to embryogenesis, including the BMP, Notch, Wnt, TNF, FGF and SHH signaling cascades [2-4]. Investigating developmental processes using tooth germ models makes it possible to follow the signaling cascades *in vitro*. The initial stage of tooth development begins with the invagination of epithelial cells from the stomodeum. These cells later form the enamel organ, which is composed of enamel epithelium, stratum intermedium, stellate reticulum and enamel matrix-secreting ameloblasts [5]. The enamel organ is bordered by ectomesenchymal tissue originating from the neural crest; that tissue produces the dental papilla [6]. The outermost layer of dental papilla faces the inner enamel epithelium and differentiates into odontoblasts, which are responsible for the formation of the dentin matrix [7]. The development of tooth germs includes several stages: initial stage starting at embryonic day 11 (E11.5), later the epithelial cells enter into the underlying mesenchyme and form bud (E13), next stage is the cap stage where the dental papilla appears (E14) and the bell stages where the cervical and lingual loop develop (E15.5-E18.5).

The continuously growing rodent incisors are showing a single cone appearance [8]. The special and unique shape of the incisor is produced by unequal growth and differentiation of the labial enamel epithelium. The enamel producing ameloblast differentiate only along the labial aspect of rodent incisor accompanied by growth of enamel only at this side, whereas odontoblast cell differentiation and dentin hard tissue formation can be obtained at both labial and lingual side [4]. It is apparent that the different signalling pathways referred above are integrated at many levels and form complex networks during rodent incisors development, as well [10].

The Toll-like receptors [TLRs] were discovered 30 years ago in relation to the development of the dorso-ventral axis in *Drosophila*, but TLRs also play a crucial role during immunity [11]. Odontoblasts express several TLRs that can modify their activity and influence the synthesis of extracellular matrix proteins [12]. Numerous studies have investigated the role of TLR signaling pathways during inflammation in dental pulp [13-14]. TLR4 is responsible for the recognition of the lipopolysaccharide [LPS], a component of Gram-negative bacterial cell wall, which is considered a specific ligand of TLR4 [15]. However, numerous heat shock proteins can activate TLRs [16-18]. TLR4 complex [MD-2, CD14 and TLR4] recognizes LPS [or another agonists] and causes the degradation of I κ B, which inhibits the nuclear translocation of NF- κ B [15]. In the absence of I κ B, NF- κ B can enter the nucleus and modify the transcription of its target genes [19]. TLR4 not only controls the immune response against oral pathogens but it is suggested that TLR4 might regulate the formation of dentin, as well as the decrease the mineralization of murine odontoblast-like cell [20-21]. It has previously been reported that TLR4-mediated signaling can decrease the mineralization of different hard tissues, such as dentin and bone, by modifying alkaline phosphatase [ALP] activity [22-23]. ALP plays a pivotal role during the mineralization of the enamel and dentin [24]. The enamel matrix undergoes biomineralization process and generates the hardest tissue in the human body [25-26].

A literature search found no experimental evidence regarding the function of TLR4 during the early stages of tooth development and mineralization of enamel matrix. We performed experiments to characterize the expression and possible role of TLR4 during tooth development.

Materials and methods

Animal care

All experimental procedures in this study followed the guidelines of the Animal Care and Use Committee of the University of Debrecen [DE FSZ/2010/10]. Pregnant females were euthanized according to these guidelines. Pregnant mice were killed by cervical dislocation and embryos were killed by decapitation. Embryonic age was estimated using the appearance of the vaginal plug (E0.5), and from their exterior features. Unique care has been taken to ensure the precise isolation of the incisor tooth germs from the lower jaw of NMRI mouse embryos at E13.5-E18.5 under Nikon SMZ 1000 stereomicroscope (Nikon, Tokyo, Japan) [9]. The fine details of the tooth germ micro-dissection procedures described below for each experimental protocol (WB, Trowell-type tissue culture and RT-qPCR), respectively.

Construction of Western Blot analysis

Western blot (WB) analysis was performed using biological triplicates from E13.5 to E18.5. Samples formed by mixture of 4-4 carefully isolated tooth germs in each time points. Mandibular incisor tooth germs remained intact, and the surface of tissue of interest did not contain any connective tissue. The WB protocol was described previously [27]. Total tissue lysates were used, and 10-20 μ g of protein was separated by 7.5 % SDS-PAGE for the detection of TLR4 and actin. A monoclonal anti-TLR4 antibody [1:200, Abcam [ab22048], Cambridge, UK] and a monoclonal anti-actin antibody [1:10,000, Sigma-Aldrich, St. Louis, USA] were used. Actin was used as a loading control. Signals were detected by enhanced chemiluminescence [Millipore, Temecula, USA] according to the manufacturer's instructions. Signals were manually developed on X-ray films.

For the detection of I κ B, the tissue of interest was sonicated in ice-cold RIPA buffer supplemented with protease inhibitors [0.1 mg/ml benzamidine, 1 mM phenyl-methyl-sulfonyl fluoride, 5 μ g/ml leupeptin, 5 μ g/ml pepstatin A, 5 μ g/ml aprotinin]. The protein content was determined using the BCA assay [Thermo Scientific, Rockford, USA], and

protein [50 µg/lane] was loaded onto 10 % acrylamide gels. The separated proteins were electrophoretically transferred onto PVDF membranes [Millipore, Bedford, USA], and the free binding sites were blocked with TBS buffer containing 10 % BSA [Sigma-Aldrich, St. Louis, USA]. After washing, membranes were incubated with the primary antibodies [anti-IκB, 1:1000, Santa Cruz, Dallas, USA and anti-β-tubulin, 1:2000 Sigma-Aldrich, St Louis, USA] overnight at 4 °C. Horseradish peroxidase [HRP]-conjugated secondary antibodies [goat-anti-rabbit-HRP, 1:1000; goat-anti-mouse-HRP, 1:1000; DAKO, Glostrup, Denmark] were applied for 4 hours at 4 °C. Finally, the immunoreactive bands were visualized with 3,3'-diaminobenzidine [Sigma-Aldrich, St Louis, USA]. Membranes were scanned by the GelCapture software and the densitometric analysis was performed with GelQuant software [Bio-Imaging Systems, Jerusalem, Israel] as previously described [28]. Briefly, the optical density values obtained from IκB in control and LPS-treated samples were normalized to the optical density values of β-tubulin bands. To provide assessment of nonspecific bands recognized by antibody of interest isotype-matched antibody has been used to blot our samples.

Organotypic tooth germ culture was used to identify the effect of LPS

Trowel-type cultures of lower incisors were prepared from E16.5 embryos [29]. This developmental stage was chosen to investigate the secretion of enamel matrix proteins based on a concentration presented in an earlier article [30]. Special attention has been paid to remove all the extra tissue surrounding the incisor tooth germs to avoid skewed data in our further analyses. The preparation of materials and dissection of tissues was carried out under sterile condition and was performed as previously described [31].

Briefly, the lower incisor tooth germs were dissected carefully from the lower jaw (mandibles) of 16,5-day-old mice (NMRI) in glass Petri dishes in PBS, using small scissors,

forceps, watchmaker forceps (Medicor, Debrecen, Hungary), and disposable 21 G needles (Terumo, Neolus, Leuven, Belgium). The dissection of the tissue piece of interest was carried out under a Nikon SMZ 1000 stereomicroscope (Nikon, Tokyo, Japan) and only the fully intact incisors pairs were used for our organotypic tooth germ cultures. Isolated tissues were placed on 0.1 µm pore-size nucleopore filters [Sigma-Aldrich, St Louis, USA] supported by metal grids in a humidified atmosphere of 5 % CO₂ in air at 37 °C. One incisor was used as a treated explant and the other as its individual control.

According to standard Trowell-type protocol (4) Dulbecco's Modified Eagle Medium (DMEM, Gibco BRL, Gaithersburg, USA) supplemented with 15 % heat-inactivated fetal bovine serum (Gibco BRL, Gaithersburg, USA) was used as the culture medium including 0.2% (v/v) PS (Gibco/Invitrogen, Paisley, UK); penicillin (10,000 IU/mL) and streptomycin (10,000 µg/mL). For the 5 days of culture, 1 µg/mL LPS-EB Biotin (derived from *E. coli* 0111:B4; InvivoGen, San Diego, USA) was added to the medium [9]. The medium was changed 48 hours after the initiation of culture (on the third day of culture). Culture was terminated on the 5th day, samples marked to E16,5+5D.

Incorporated LPS was detected by immunohistochemistry

Samples were fixed in Sainte-Marie fixative for 30 min at 4 °C after *in vitro* culturing. Whole-mount tooth germs were incubated by Vectastain Elite ABC Kit [Vector Laboratories Ltd., Peterborough, UK] to label the incorporated LPS-EB biotin. Control samples were stained using the same procedure but with PBS replacing the Vectastain Elite ABC Kit. We visualized LPS-EB biotin with DAB [Vector Laboratories Ltd., Peterborough, UK] for conventional light microscopy. No specific signal was recorded from control sections. DAB immunoprecipitation was evaluated independently by 3 researchers using immunostained samples from each individual case.

ELISA and alizarin red staining were used to determine mineralization of hard tissues

We followed the previously described method [25], with slight modifications. Briefly, tooth germs were mechanically homogenized in Tris-glycine buffer containing 1 % SDS and protease inhibitors. Protein content of the samples was measured using the BCA assay. NUNC Maxisorp plates [Nunc Intermed, Copenhagen, Denmark] were coated with 10 µg protein/well in coating buffer [15 mM Na₂CO₃, 35 mM NaHCO₃, 0.02 % NaN₃, pH=9,6]. Free binding capacity of the polystyrene surfaces was blocked with 1 % BSA followed by anti-collagen X primary antibody [Abcam, Cambridge, UK]. The immunoplates were treated with HRP-conjugated goat-anti-rabbit IgGs secondary antibody [DAKO, Glostrup, Denmark]. The color reaction was developed with o-phenylene-diamine substrate and absorbency was measured at 492 nm with a microplate reader.

During the alizarin red staining, tooth explants were fixed in Saint-Marie fixative for 2 hours, and after washing in deionized water, explants were stained with alizarin red [Sigma Aldrich, St. Louis, MO, USA] for 10 min on room temperature. Excess dye was removed, and the samples were washed with 20 % glycerol for 5 minutes at room temperature.

Total RNA preparation, reverse transcription and reverse transcription couples quantitative PCR [RT-qPCR] and whole-mount *in situ* hybridization

To be able to handle the apical portion of incisor tooth germs separately from their CL region, the lower mandibular incisor has been removed from each hemi-mandibles like same way as described above, but after isolation of tissue of interest the apical edge of incisor has been micro-dissected from cervical loop (CL) region. During this microsurgery the CL region has been removed gently using size 4 forceps and scalpel. Finally the CL has been fall apart while the tissue of interest remained intact, which was immediately placed for RT-qPCR experiments. RT-qPCR was performed as described in [32] with minor modifications. Briefly,

total RNA was prepared using Trizol reagent [*Invitrogen*] according to the manufacturer's instructions. cDNA was synthesized from 200 ng RNA sample using High Capacity cDNA Reverse Transcription Kit [Applied Biosystems, Foster City, CA, USA]. qPCR reactions contained 10 ng cDNA obtained as described above. For control and for the treated group analysis a mixture of the apical incisor part of the 7 littermate tissues of interest has been collected and used for RT-qPCR. The quantitative PCR reactions were performed using the LightCycler 480 system [*Roche*, Basel, Switzerland] and a qPCR supermix [*PCRBiosystems*] with the following primers: ameloblastin [fwd: 5'- CATGCAGGACTTCTTGCTTTC; rev: 5'-GGTGCACCTTTGTTTCCAGGTA] and cyclophilin as control [fwd: 5'-TGGAGAGCACCAAGACAGACA; rev: 5'-TGCCGGAGTCGACAATGAT], then ameloblastin expression was normalized for cyclophilin expression. In all cases qPCR reactions were performed on untranscribed RNA to verify the absence of genomic DNA contamination and data presented were obtained from three independent experiments.

Non-radioactive probes were used during *in situ* hybridization, according to the Roche protocol [Roche, Mannheim, Germany], with Dig-labeled ameloblastin probes obtained using *in vitro* transcription from PCR templates [33]. Probes were purchased from Integrated DNA Technologies [Iowa, USA]. - T3 flanked sense: 5'-AATTA ACCCT CACTA AAGGC AGAAG GCTCT CCACT GCAA-3'; - T7 flanked antisense: 5'-TAATA CGACT CACTA TAGGA GCAGT CAGGGT TTTCC ACC-3'.

Analysis of data and image capturing

Photomicrographs were taken using a Nikon Eclipse E800 microscope [Nikon Corporation, Tokyo, Japan], images were processed using Adobe Photoshop CS4 [Adobe Systems Inc., San Jose, CA, USA]. For statistical analysis of ELISA and I κ B degradation [WB], at least 3 individual samples from 3 different culture groups were used. Where applicable, data are

expressed as mean \pm SEM. Statistical analysis was performed using Student's *t*-test, and differences were considered significant at $p < 0.05$. Statistical analysis was paired *t*-test at the case of RT-qPCR [$p < 0.05$].

Results

TLR4 is expressed from the cap stage of tooth development and can be activated by LPS

Protein expression of TLR4 was studied with Western blot analysis in developing lower mouse incisors E13.5 to E18.5 stages (about 75 kDa) [Fig 1A]. TLR4 was not detected by Western blotting from E13.5 tooth germs. Administration of LPS resulted in a significant reduction in I κ B in E16.5 tooth germs compared to control samples [Fig 1C]. The results were normalized to the levels of tubulin.

Biotin-bound LPS labeled functionally active TLR4 in the enamel organ and in preodontoblasts

Whole-mount samples showed strong immunoreactivity for LPS in the labial part of the treated E16.5+5D tooth germs. After histological processing of samples, the presecretory and secretory ameloblasts, stratum intermedium and preodontoblasts obviously contain biotin-labeled LPS in the tooth germs [Fig 2, inserts A-B]. In the region of postsecretory transition, signals in ameloblasts decreased. Because these structures contain active TLR4, LPS appears to be a specific ligand of TLR4.

LPS treatment decreased mineralization of tooth germ but did not affect the morphology of E16.5 tooth germs

No morphological alterations were detected in tooth germs after LPS administration [Fig 3 A table]. After culturing, we used alizarin red staining to detect the mineralization process [Fig 3 B table, A-B]. In control samples, strong extracellular Ca²⁺ deposits were present in the dentin and enamel. LPS-treated tooth germs showed significantly lower Ca²⁺ content in the

same structures. LPS administration resulted in weak mineralization only on the labial side of the enamel organ. LPS-treated tooth germ showed no obvious mineralization on either the lingual or labial side. ELISA on LPS-treated and control total tooth germ samples showed a significant decrease in the level of type X collagen upon LPS administration in the culture medium, corroborating the results of the alizarin red staining [Fig 3 B table, C].

LPS treatment increases the maturing of ameloblasts

To investigate a possible effect of TLR4 on ameloblasts, we examined the expression of ameloblastin RNA using *in situ* hybridization and RT-qPCR. Ameloblastin is a specific marker for secretory ameloblasts at E16.5 and correlates well with the maturation stage of these cells. Notably, the presence of ameloblastin was visible only in the enamel in both control and LPS-treated cultures [Fig 4 inserts A-B]. To qualify the obvious differences between the amount of ameloblastin mRNA in the treated and control group, RT-qPCR were performed. Significant higher ameloblastin expression was detected in LPS-treated than samples in control samples [Fig 4 inserts C] [$p < 0.005$].

Discussion

TLRs play roles in developmental processes of the derivation of ectoderm [34, 16], but there is limited information about their role during embryonic development. TLRs in the tooth have been widely investigated during pathological cases; however, until now, we did not know any of their biological functions during tooth development. We used an exogenous specific ligand of TLR4 to investigate the effect of TLR4 during tooth development, but several endogenous ligands can activate the TLRs, including Hsp-s [17]; Gp96; high mobility group proteins; and proteoglycans [35-37]. This study provides the first evidence that TLR4 may have function in tooth development. We found that TLR4 was continuously expressed from the cap stage E14,5 of tooth development. The ultrapure LPS [a potent specific agonist of TLR4] may

cause the degradation of I κ B, the negative inhibitor of the NF- κ B signaling pathway. This result indicates functional activity of TLR4 during tooth development [38]. The possible role of MAPK signaling during tooth development remains an open question, and this pathway is also activated by TLR4 [39]. The applied ultrapure LPS is a specific ligand of TLR4, however non purified LPS can activate TLR2. In a screen of the TLR2 expression profiles in an online database [Allen Institute for Brain Science, Allen Developing Mouse Brain Atlas, available at <http://developingmouse.brain-map.org>], we can declare that TLR2 did not express during the bell stage of the tooth development. In contrast **to earlier studies** of TLR4 KO where **authors** could investigate the absent of function, we used temporally activation of TLR4 in order to simulate the effect of TLR4. The activation of TLR4 receptor did not alter the morphology of the tooth germ. Several studies have investigated the role of TLR4 signaling in relation to dentin mineralization of the mature tooth [40-41]. According to the literature, LPS treatment can decrease the mineralization of the hard tissues through the modification of non-tissue-specific alkaline phosphatase activity [13, 24]. Lower incisors of E16,5 NMRI mice has been used *in vitro* culturing to quantitatively analyse the ameloblastin expression level at the end of 5 days long culturing, in order the screen the effects of LPS on function of the secretory ameloblasts. According to earlier studies at this stage, the enamel matrix proteins (amelogenins and non-amelogenins) are already present influencing the early steps of enamel matrix synthesis. Ameloblastin concentration can affects the mineralization processes and expression level of AMBN shows a negative correlation with the mineralization status of the enamel matrix (5). At the end of the 5th days of the Trowel-type incisor culturing we found obvious differences between the treated and control E16.5+5D incisor tooth germs using alizarin red staining. We propose two possible reasons: first, the downstream signaling pathway of TLR4 may decrease the ALP activity of dental cells and osteoblasts; second, LPS may bind Ca²⁺ that inhibits the incorporation of the mineral phase into the dentin and enamel

matrix [40-41]. The absence of free Ca^{2+} ions has been reported to inhibit ameloblast maturation [42]. The absence of free Ca^{2+} ions contradicts by the result of *in situ* hybridization and RT-qPCR, which showing the acceleration of ameloblast maturation. The biotin-conjugated LPS labeled the active TLR4, which was present in presecretory and secretory ameloblasts, stratum intermedium and preodontoblasts. These structures normally contain ALP and participate in the mineralization of the tooth germ; we observed decreased ALP activity in our samples [data not shown] [42]. Histochemical staining results were supported by ELISA, which showed a decreased amount of collagen type X, which plays a principal role during ossification and the formation of an optimal environment for hydroxyapatite crystal deposition [25]. Collagen type X is present exclusively in the enamel matrix in tooth germs, indicating a weaker mineralization process in the enamel matrix [25]. To detect the possible function of TLR4 receptor in the maturing of ameloblasts, we performed *in situ* hybridization and RT-qPCR against ameloblastin mRNA. The ameloblastin indicates the maturation of the ameloblasts during tooth development and forms a part of the enamel matrix, however transiently it **is** expressed in dentin matrix [5]. The whole mount *in situ* hybridization indicates that ameloblastin originates only from ameloblasts. The LPS treated samples showed accelerated synthesis of ameloblastin mRNA, indicating a role for TLR4 during ameloblast differentiation. Previous papers described a similar effect of TLR4 on odontoblasts, showing that TLR4 activation increases the synthesis of dentin and decorin, matrix proteins related to LPS treatment, and regulate odontoblast differentiation [43]. Accelerated ameloblast maturation may be a direct effect of TLR4 signaling or may be a compensatory mechanism of the inhibition of mineralization. We did not decipher which downstream TLR4 signaling pathway causes these changes during tooth development warranting further investigations. It is also of interest which endogenous ligand[s] activate TLR4 during development.

Conclusion

This study reveals the possible role for TLR4 during the synthesis and the mineralization processes of enamel matrix. The TLR4 receptor is expressed continuously from the beginning during the bell stage of tooth development. To verify the function of TLR4, we detected the degradation of I κ B after LPS treatment. Biotin-labeled LPS was present in the enamel organ and preodontoblasts and decreased the mineralization of dental tissues. The treatment of tooth germ with LPS-EB biotin did not cause any obvious morphological differences between our samples, but it did positively regulate ameloblast maturation, however the mineralization of tooth germs was weaker. Our results suggest that several molecules can modify the mineralization of tooth, which can be valuable information to understand congenital tooth anomalies.

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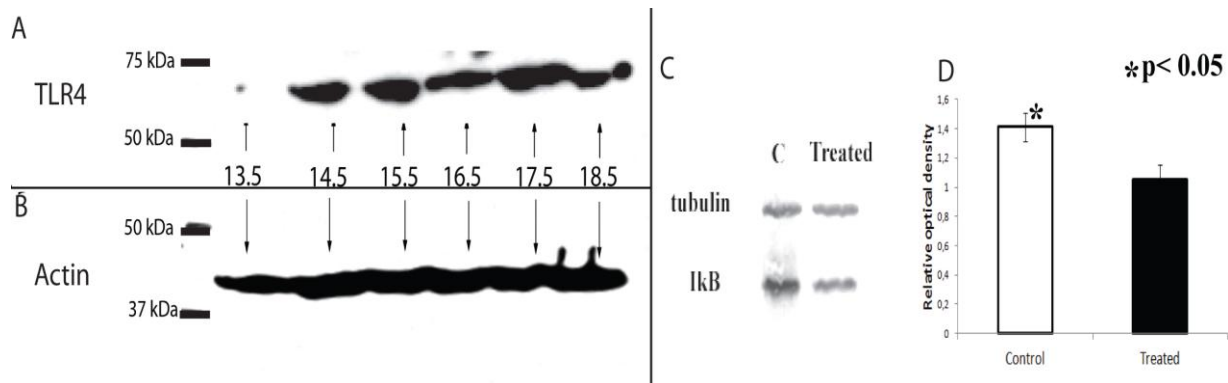
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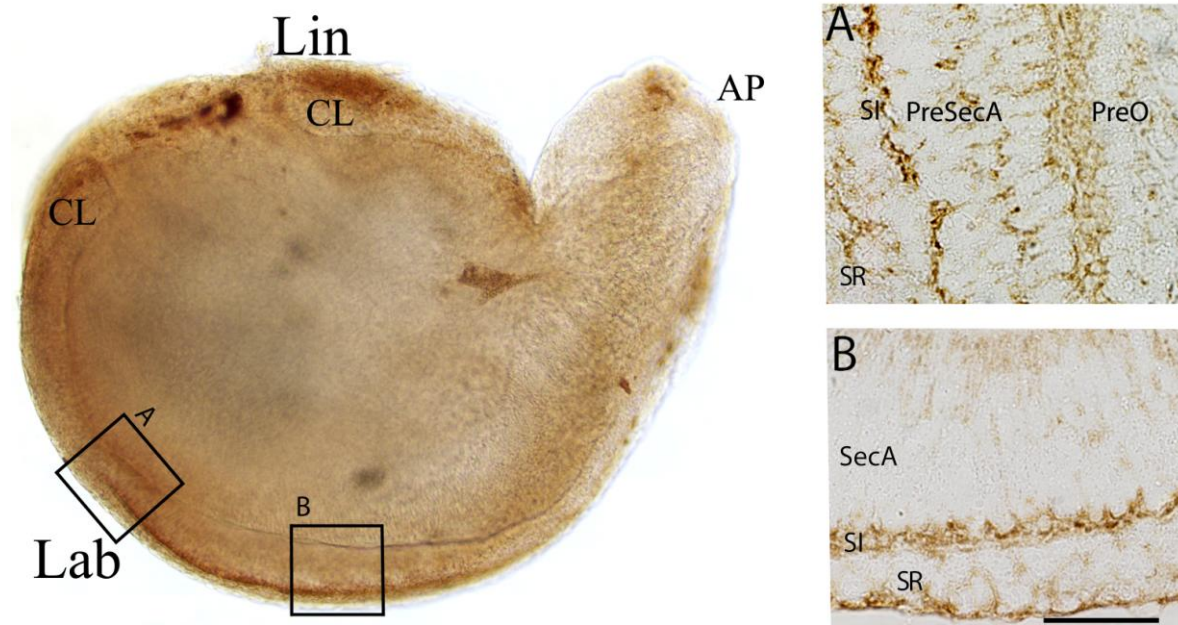
Figure legends

Figure 1. TLR4 is expressed from the cap stage of tooth development, and LPS treatment decreases the amount of IκB.



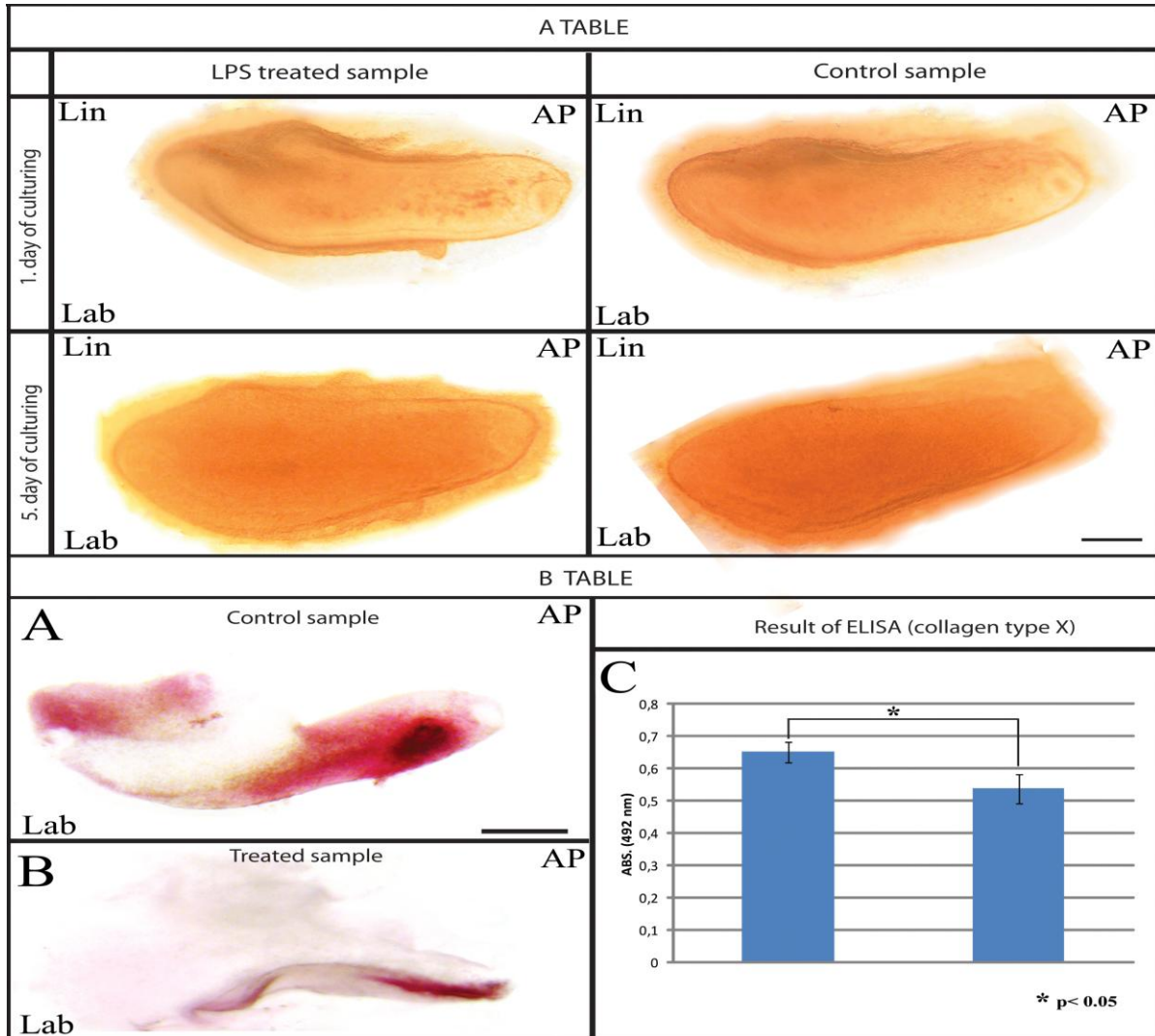
By WB, we determined that TLR4 present continuously from E14.5 to E18.5 during tooth development [n: 3] [inset A]. Inset B shows loading control [actin]. LPS treatment decreased the amount of IκB during the *in vitro* culturing during the bell stage (E16.5), control was tubulin C inset. D inset shows the calculated optical density from the treated and control groups [Student's t-test; $p < 0.05$, n: 6-6]. Data represent the mean of optical density \pm SD [inset D].

Figure 2. Structures of enamel organ and preodontoblasts take up LPS.



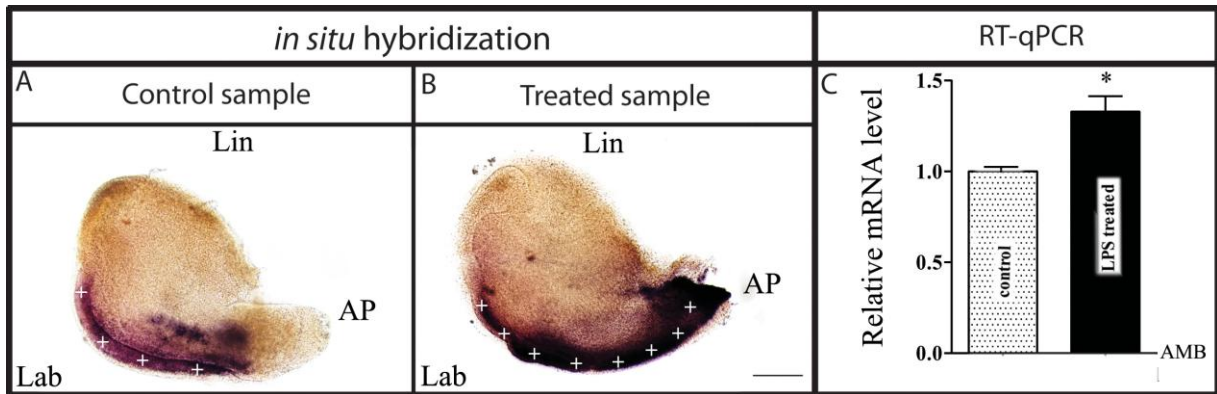
Whole-mount immunohistochemical sample showing strong immunoreactions in the labial part of tooth germs [representative image of 3 experiments is shown]. At higher magnification [**inset A**], the presecretory ameloblasts [PreSecA], preodontoblasts [PreO], stratum intermedium [SI] and stellate reticulum [SR] contain biotin labelled LPS. **Inset B** shows immunoreactions in the basal part of secretory ameloblasts [SecA], stratum intermedium [SI] and stellate reticulum [SR]. Abbreviations: Lab, labial side; Lin, lingual side; CL, cervical loop; AP, apical side. Scale bar represents 50 μm in the overview image, and 20 μm in panel A and B.

Figure 3. The activation of TLR4 does not alter the morphology of the developing tooth but inhibits the mineralization of hard tissues.



On the fifth day of culture, we did not find any obvious morphological alteration between our samples [Table A]. Alizarin red staining indicates differences between the mineralization status of our groups [Table B, inset A-B]. The amount of type X collagen was determined by ELISA [Table B, inset C], data represent the mean of absorbance [ABS] ± SD, and the difference is significant [Student's t-test; p<0.05, n: 8-8]. Abbreviations: Lab, labial side; Lin, lingual side; AP, apical side. Scale bar: 100 µm.

Figure 4. LPS alters the maturation of ameloblasts at E16.5+5D.



Whole mount *in situ* hybridization indicates ameloblastin mRNA in the enamel organ in the control sample [inset A] and in the treated sample [inset B]. Crosses (+) are indicating the tissue of interest expressing ameloblastin. Inset C: Real-time-qPCR bar graphs showing alterations of relative ameloblastin mRNA expression in the control and in the LPS treated tissue of interest after 5 days long culturing, asterisks indicate significant differences [paired *t*-test; $p < 0.05$, [n: 7-7]] between LPS treated and control samples. Data are expressed as the mean \pm SEM. Abbreviations: Lab, labial side; Lin, lingual side; AP, apical side; AMB, ameloblastin. Scale bar: 50 μ m.