

Up-regulation of TLR2 and TLR4 in high mobility group Box1-stimulated macrophages in pulpitis patients

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ABSTRACT

Objective(s): High Mobility Group Box1 (HMGB1) is a nonhistone, DNA-binding protein that serves a crucial role in regulating gene transcription and is involved in a variety of proinflammatory, extracellular activities. The aim of this study was to explore whether HMGB1 stimulation can up-regulate the expression of Toll-like Receptor 2 (TLR2) and Toll-like Receptor 4 (TLR4) on macrophages from pulpitis and to clarify the subsequent events involving Th17 cells and Th17 cell-associated cytokine changes.

Materials and Methods: Having prepared dental pulp tissues of pulpitis and healthy controls, macrophage were isolated and cultured. Macrophages were thereafter stimulated by HMGB1 time course. RT-QPCR, flowcytometer, immunofluorescence, Western blotting, and ELISA techniques were used in the present research.

Results: Our results showed that the expression of TLR2 and TLR4 on macrophages stimulated with HMGB1 increased in pulpitis compared with controls (macrophages without HMGB1 stimulation) with a statistical significance ($P < 0.001$). In addition, the levels of IL-17, IL-23, and IL-6 in supernatants from cultured macrophages stimulated with HMGB1 from pulpitis increased, and NF- κ B, the downstream target of TLR2 and TLR4, also showed a marked elevation after macrophages' stimulation by HMGB1.

Conclusion: The evidence from the present study suggests that the enhanced TLR2 and TLR4 pathways and Th17 cell polarization may be due to HMGB1 stimulation in pulpitis.

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Introduction

The defense system of dental pulp consists of innate and adaptive immune systems (1). In addition, dental pulp possesses many MHC II-positive cells, which are very effective antigen-presenting cells for initiating immune responses against oral microorganisms (2). Macrophages are a heterogeneous population of antigen-presenting cells characterized by their morphology, function, and metabolism (3, 4).

Despite the fact that exudative macrophages migrate to sites of inflammation in response to several chemokines, resident macrophages are ubiquitously distributed around the body under normal healthy conditions (5, 6). Large numbers of macrophages exist in dental pulp and are considered to be the primary immunocompetent cells that fight against bacterial infections caused by dental caries. In fact, macrophages migrate toward the infection site in pulpitis (7, 8).

Macrophages express various pattern-recognition receptors, such as Toll-like receptors (TLRs) to recognize

targets, and show high phagocytic potential (9). Activation signaling through TLRs induces a release of various cytokines and mediates innate immune responses through regulating phagocytosis and triggering antimicrobial activity (10). T cells are present in all pulp tissue areas in intact teeth, whereas B cells are not seen in normal pulp tissues (8). In this regard, TLRs may be crucial cellular sentinels in detecting endangering signals such as High-mobility group box 1 (HMGB1) during inflammation. HMGB1, found to be an important cytokine mediating the response to infection, injury, and inflammation has been called "nuclear weapon" in the immune arsenal (11).

It is a nonhistone, DNA-binding protein that serves a crucial role in regulating gene transcription and is involved in a variety of proinflammatory, extracellular activities (12). HMGB1 is implicated in the pathogenesis of many inflammatory diseases including pulpitis (13).

The present study explores the up-regulating of TLR2 and TLR4 via HMGB1 stimulation in pulpitis.

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Materials and Methods

Reagents

TLR2 and TLR4 primers were obtained from Shanghai Sangon Company, Shanghai, China. Recombinant human IL-17, IL-6, and IL-23 ELISA kits were purchased from Bender Med System, Vienna, Austria. Fluorescein Isothiocyanate (FITC)-conjugated rabbit Anti-human CD16 mAb (Leu M3; Becton Dickinson, Franklin Lakes, NJ, USA), PE-conjugated Anti-human TLR2 mAb (Leu11; Becton Dickinson), PE-conjugated Anti-human TLR4 mAb (Leu11; Becton Dickinson), PE-labelled anti-human IgG, rabbit Anti-human CD45 antibody labelled with PIPE, and goat Anti-human IL-17 antibody were purchased from Invitrogen Company, Hercules, CA, USA. HMGB1 was obtained from (Invitrogen Company, USA), a gene recombinant product, whose purity was more than 93% as determined by SDS-PAGE, without DNA, and the Lipo-polysaccharide (LPS) was less than 1.0 EU per Ig (10) of the protein as determined by the Limulus ameocyte lysate (LAL) method. LPS (*Escherichia coli* serotype 055:B5) was purchased from Worthington (Lakewood, NJ, USA).

The preparation of dental pulp tissue specimens

Dental pulp tissues were collected from extracted third molars of 20 patients aged 20–28 years who underwent routine surgical or endodontic treatment at the Department of Endodontic and Maxillofacial Surgery, School of Dentistry, Tabriz University of Medical Science, Tabriz, Iran. The samples were prepared separately. Ten controls were obtained from the healthy pulps of the abovementioned teeth. We observed the Declaration of Helsinki for all procedures in the present study. None of the patients had any clinically significant medical history of any diseases or took any medications. The inflamed pulp tissues were collected from patients with symptoms of irreversible pulpitis such as response to cold or heat stimulus (6).

Non-carious healthy pulp tissues were used as controls. The pulps were extirpated using a sterilized barbed broach or Hedstrom hand file. For teeth diagnosed with inflamed pulps, rubber dam isolation was obtained and the tooth and rubber dam were disinfected by using 0.2% chlorhexidine gluconate. The carious teeth structures were removed to access the root canal system. Pulp tissues were collected using a sterilized barbed broach or Hedstrom hand file. Pulp tissues were gently separated from the instrument and placed in a sterile Eppendorf tube. One section of each specimen was stained with hematoxylin and eosin (H&E). All samples were stored at -80 ° C until processing.

Isolation and culture of macrophages

Macrophages were isolated from pulpitis tissues. Pulpitis tissues were kept directly on ice after resection, and the isolation procedure was started within 15 min. Specimens were weighed and cut into small tissue fragments (1 to 2 mm) in Gey's balanced salt solution

(GBSS). Tissues were thereafter incubated in 75 ml GBSS with 0.025 g collagenase type-1 on a magnetic stirrer at 37 °C with continuous pH 7. After 10 mins, the suspension and the remaining fragments were filtered through gauze (pore 60 pm).

The suspension was centrifuged at 100g for 5 mins and washed twice by suspending the pellet in 50 ml GBSS and 0.8 pg/ml DNase. Subsequent to washing, the pellets were suspended in 5 ml GBSS and 0.8 pg/ml DNase. The non-parenchymal cells were separated from nonviable cells, the remaining parenchymal cells, and erythrocytes by centrifugation on a 16% Nycodenz (Nycomed AS, Oslo, Norway) gradient for 20 mins, 300g at 4°C. The low-density fraction was collected, re-suspended in 10 ml GBSS, 0.8 pg/ml DNase, and centrifuged at 100g for 5 mins. The final pellets were re-suspended in 5 ml GBSS and 0.8 pg/ml DNase. Cells (4×10^6 cells [isolated from each third molar] /well in a 6-well plate) were plated in tissue culture plates at 37°C for 30 mins before washing and incubating in tissue culture media (DMEM media) containing 5% FBS overnight. Approximately 53% and 38% of these cells were macrophages in pulpitis and healthy controls, respectively, as estimated by their ability to ingest latex beads.

Macrophages were isolated separately from each tooth and we selected tissues with at least 4×10^6 cells. Thirty wells for cases and controls (each well with 8×10^6) were used per experiment. Experiments were done with macrophages from one tooth in each well. The other cells were other phagocytes and lymphocytes.

Cell viability was always 90%+SD as assessed by trypan blue. All the experiments were subsequently performed after washing the cells three times with serum-free media. For each experiment, the macrophages were isolated from pulpitis and healthy pulp tissues. The cells were cultured in RPMI1640 cell culture buffer.

Cell culture and HMGB1 stimulation

The macrophages isolated from the pulpitis and healthy pulps were cultured at 1×10^6 cell/ml in RPMI-1640 medium (GIBCO, Invitrogen Corporation, UK) supplemented with 10% fetal bovine serum, and 1% streptomycin/penicillin. The cell suspensions were plated in 6-well culture plates and stimulated with 0.5 µg/ml of HMGB1 for 2, 4, 8 and 12 hr. For blocking TLR2 and TLR4 or HMGB1, 10 lg/ml rabbit Anti-human TLR2 and TLR4 or Anti-HMGB1 antibody (Abcam, Cambridge, UK) was added 1 hr before treatment with HMGB1. The cell suspensions without HMGB1 stimulation were used as controls.

Quantitative real-time PCR (qRT-PCR)

The expressions of TLR2 and TLR4 were measured by quantitative real-time polymerase chain reaction (qRT-PCR), and all samples were calibrated by b-actin. Briefly, total RNA was isolated from macrophages using the TRIzol (Invitrogen) isolation solution according to the manufacturer's instructions. Isolated RNA was eluted in RNase-free water and reverse-transcribed

with ReverTra Ace RT-qPCR kit (TOYOBO, Osaka Boseki, Japan).

The TLR2 and TLR4 mRNA levels were quantified by qRT-PCR amplification using a 7500 fast real-time PCR system (Applied Biosystems, Foster, CA, USA) in a total volume of 10 μ l, containing 5 μ l SYBR Green1 mix (Bio-Rad, Hercules, CA, USA), 0.4 μ l forward and reverse primers and 0.06 μ l Tag polymerase, 2.5 μ l ddH₂O and 2 μ l cDNA templates.

The recommended cycling conditions for qRT-PCR were as follows: denaturation at 94 °C for 2 min followed by 35 cycles of 94 °C 10 sec, 60 °C 15 sec, and 72 °C 30 sec. The specificity of the amplification products was controlled using a melting curve analysis. The copy number of the objective gene or b-actin transcript in the samples was calculated with the BIO- SYSTEM software according to corresponding standard curves.

Our designed primers were as follows: TLR4 forward primer, 5'-tgtgcaaaactgcccgggagga-3', TLR4 reverse primer 5'-acttctccttcagcttggcagc-3', TLR2 forward primer, 5'-ttgtcccgtgcaaaactgcccgggagga-3', TLR2 reverse primer, 5'-aagtcctgtattactgcccgttagga-3'; b-actin forward primer, 5'-tggatcctgtggcatcatgaac-3', b-actin reverse primer, 5'-taaacgcagctcagtaacagtcgc-3'. These primers have exon-exon boundaries however our introns were large enough not to interfere with the PCR and not result in a double melting peak. Each gene was amplified in triplicate. The applicant length of ~100 bp was optimal for use with SYBER Green when running qPCR (Figure 1).

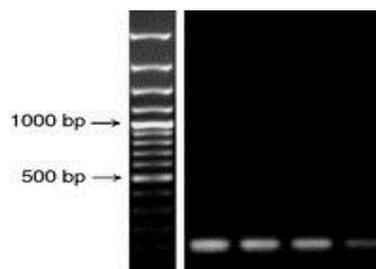
Flowcytometer analysis

The macrophage cell suspensions were washed with RPMI 1640 medium (Life Technologies, Gaithersburg, MD, USA) and suspended in phosphate-buffered saline (PBS) with 1% heat-inactivated fetal calf serum (FCS; Life Technologies).

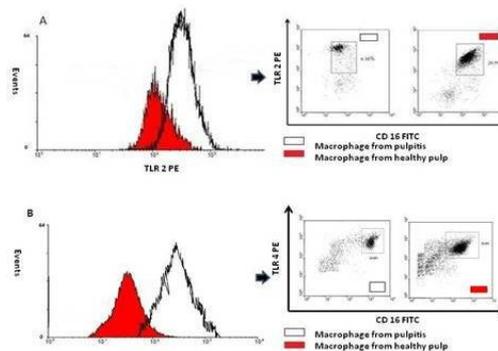
Cell surface expression of TLR2 and TLR4 was analyzed by cell surface staining and flow cytometric analysis. Briefly, macrophage suspensions were incubated with PE-conjugated rabbit Anti-human TLR2 and TLR4 monoclonal antibodies (mAb), and the cells were washed and then incubated with fluorescein isothiocyanate (FITC)-conjugated rabbit Anti-CD16 mAb. After washing, the cells were re-suspended in 1% FCS / PBS. Analyses were performed on a flow cytometer (Becton Dickinson), and the macrophages were specifically analyzed by selective gating based on parameters of forward and side light scatters.

In each sample, the number of events was measured as the events were gated on a CD16-FITC versus CD4-RPE dot plot. The total number of calibration fluorescence particles was counted while the events from RPE-Cy5 versus forward angle light scatter parameters were gated. The absolute CD16+ count was equal to the ratio of CD16+ cells counted to the number of calibrated particles counted, multiplied by the concentration of the calibration particles considering the fact that the concentration of the calibration particles was known. The results were expressed as mean fluorescence intensity units (MFI)/10⁵ cells.

1-A



1-B



1-C

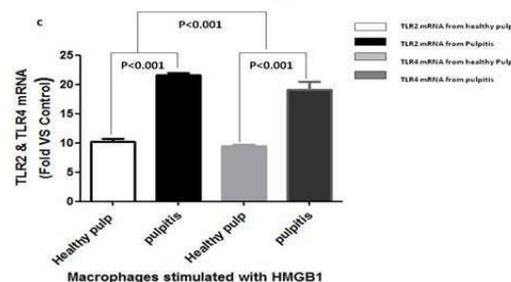


Figure 1. A: The amplicon sizes of qPCR. B: TLR2 and TLR4 expressions on CD16+ cells. (A) The expression of TLR2 on CD16+ cells analyzed with FACS. The level of TLR2 on macrophages from pulpitis was higher than that from healthy pulp, $P < 0.001$. (B) The expression of TLR4 on CD16+ cells analyzed with FACS. The level of TLR4 on macrophages from pulpitis was higher than that from healthy pulp, $P < 0.001$. (C) The mRNA of TLR2 and TLR4 on CD16+ cells determined by QRT-PCR, the values were expressed as the increased fold compared with b-actin, the TLR2/b-actin mRNA increase showed that the TLR2 expression was increased in macrophages from pulpitis compared with healthy pulps ($P < 0.001$). The diagrams were prepared using Corbett real-time PCR and FACS and SPSS software. The results were normalized by Actin B values and then pulpitis samples compared to healthy controls

Immunofluorescence staining

Immunofluorescence staining of paraffin-embedded macrophages from pulpitis and healthy pulp were performed as described previously (14). By means of washing, deparaffinization, rehydration, and antigen unmasking were carried out for the cells. Samples were then immersed in blocking buffer for 60 mins; and primary antibodies Anti-CD4 and Anti-IL-17 (Santa Cruz Biotechnology, USA) were applied for 2 hrs at room temperature. After washing, FITC- and PE-labeled

secondary antibodies were added for 1 hr. Then the macrophages from pulpitis were stimulated with HMGB1 for 12 hrs, and then they were incubated with Anti-human IL-17 antibody for 2 hrs at room temperature. After washing, the PE-labeled secondary antibody (PE-labeled Anti-human IgG) was added and re-incubated for another hr. Sections were viewed with a fluorescent [or fluorescing] microscope (Olympus, Japan) and calculated using the Image J software.

Western blot analysis

Macrophages from pulpitis and healthy pulps which were treated with HMGB1 were lysed and then electrophoresed on 12% SDS-PAGE gels and transferred onto polyscreen PVDF transfer membranes (PVDF; PerkinElmer, USA). Membranes were blocked with 5% (w/v) non-fat dry milk, 1% (v/v) Tween 20 in PBS for 1 hr at room temperature and incubated overnight with commercially available Anti-Nf-KB antibody (1:1000) (Abcam, USA) at 4 °C. Detection was performed with electrochemiluminescence (ECL) and the blots were quantified by densitometry using an image analysis program (Amercontrol Biosciences, USA).

Enzyme-linked immunosorbent assay

The concentrations of IL-6, IL-23, and IL-17 from cell culture supernatant stimulated with HMGB1 were determined using commercially obtained enzyme-linked immunosorbent assay (ELISA) kits (Bender Med Systems, Vienna, Austria) according to the manufacturer's instructions. The typical detection ranges for ELISA are 0.1 to 1 mole or 0.01 ng to 0.1 ng, with sensitivity dependent upon the particular characteristics of the antibody-antigen interaction. The detection limits of cytokine kits are 1 pg/ml.

Statistical analysis

All statistical analyses were performed using SPSS 17.0 statistical analysis software. Data are shown as the mean±SD in the text and figures. Comparisons between paired and unpaired groups were performed using the t-test or one-way ANOVA with Bonferroni correction < 0.05 considered to be statistically significant. The diagrams were prepared using Corbett real-time PCR, FACS, and SPSS software packages.

Results

TLR2 and TLR4 expressions on CD16+ cells from pulpitis

The expression of TLR2 and TLR4 was enhanced on CD16+ macrophages from pulpitis stimulated with HMGB1 compared with controls at a rate that was statistically significant (Figures 1A, B). In addition, qRT-PCR results showed that the expression of TLR2 and TLR4 mRNA in CD16+ macrophages from pulpitis was significantly higher than that from healthy pulps (Figure 1C).

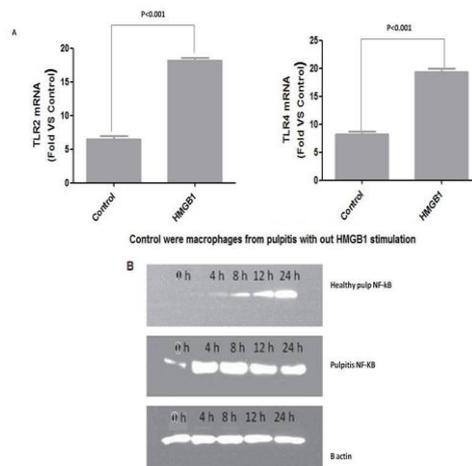


Figure 2. The effect of HMGB1 stimulation on macrophages. (A) The macrophages stimulated or unstimulated by HMGB1 *in vitro* were both from pulpitis, the levels of TLR2 and TLR4 mRNAs in macrophages were determined by qRT-PCR. The TLR2 and TLR4 / b-actin mRNA increase showed the expression of TLR2 and TLR4 was obviously increased in macrophages from pulpitis stimulated by HMGB1, compared with unstimulated cells ($P < 0.001$). (B) Western blotting showed that the expression level of NF- κ B in macrophages from pulpitis stimulated by HMGB1 was also enhanced compared with healthy pulps, especially while stimulated with HMGB1 for 4 and 8 hrs. Representative gel was shown with b-actin as a loading control

TLR2 and TLR4 expression on macrophages stimulated with HMGB1 *in vitro*

The macrophages were cultured and stimulated with HMGB1, and then mRNA levels of TLR2 and TLR4 were measured by qRT-PCR. As shown in Figure 2A, the mRNA of TLR2 and TLR4 were increased in macrophages from pulpitis stimulated with HMGB1. To explore whether the TLR4 and TLR2-mediated nuclear factor-kappa B (NF- κ B) signaling pathway contributes to the inflammatory process of pulpitis, the expression level of NF- κ B, the downstream target of TLR2 and TLR4, in the macrophages from pulpitis stimulated by HMGB1 was detected using Western blot, and the result showed that it was obviously increased (Figure 2B), especially when macrophages were co-cultured with HMGB1 for 8 hrs later.

Cytokine concentrations in culture supernatant of macrophages stimulated by HMGB1

The concentrations of cytokines were detected, and the results indicated that the levels of IL-17, IL-6, and IL-23 were significantly increased in the case of HMGB1 stimulation for 8 hrs (Figure 3A). To illustrate the effect of HMGB1, Anti-TLR2 antibody or Anti-TLR4 Antibody (10 ng /ml) was added into the system of macrophage culture 1 hr before treatment with HMGB1, and the cells' supernatant was collected for ELISA.

The data showed that the Anti-TLR2 antibody and Anti-TLR4 antibody could significantly decrease the expressions of IL-17, IL-6, and IL-23 (Figure 3A). To illustrate the specificity of HMGB1 function, LPS was compared with HMGB1 effect in stimulating IL-17

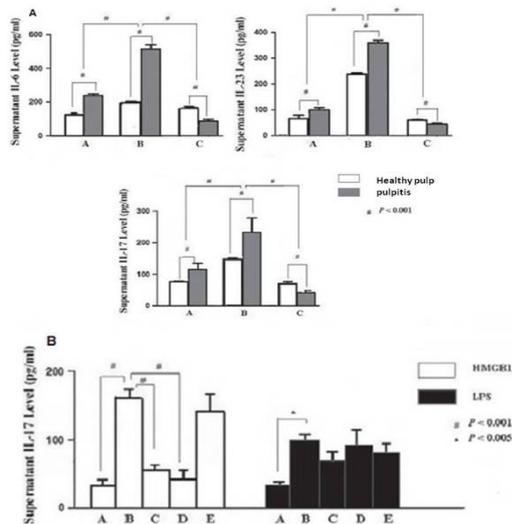


Figure 3. supernatant levels of IL-6, IL-23, and IL-17. (A) The culture supernatants of macrophages from pulpitis or healthy pulp without HMGB1 stimulation; (B) The culture supernatants of macrophages from pulpitis or healthy pulps stimulated with HMGB1 for 8 hrs *in vitro*; C: Based on B, the Anti-TLR2 antibody was added to the system of macrophage culture 1 hr before treatment with HMGB1. D: Based on B, the Anti-TLR4 antibody was added to the system of macrophage culture 1 hr before treatment with HMGB1. The levels of IL-17, IL-6, and IL-23 in culture supernatants of macrophages from pulpitis and healthy pulps were significantly increased in the case of HMGB1 stimulation for 8 hrs, compared with HMGB1-unstimulated cells, particularly in cultured supernatants of macrophages from pulpitis. Anti-TLR2 and Anti-TLR4 antibodies could significantly inhibit the expression of IL-17, IL-6, and IL-23, while the antibody was added into the cultured macrophages from pulpitis or healthy pulps ($P < 0.001$). In B, to illustrate the specificity of HMGB1 function, LPS is compared with HMGB1 effect in stimulating IL-17 secretion on macrophages from healthy pulps. (A) The cultured supernatants of macrophages without any stimulation; (B) the cultured supernatants of macrophage stimulated by HMGB1 or LPS for 8 hrs *in vitro*; based on B, the Anti-TLR2 and Anti-TLR4 antibodies (C), Anti-HMGB1 antibody (D), and rabbit serum IgG as a control antibody (E) were added into the system of macrophages culture 1 hr before treatment with HMGB1 or LPS. The Anti-HMGB1 data are not shown

secretion on macrophage from controls. TLR2 and TLR4 or blocked HMGB1 affected IL-17 secretion in HMGB1 stimulation group but did not significantly affect IL-17 secretion in LPS stimulation groups (Figure 3B). Moreover, the levels of IL-17, IL-6, and IL-23 were significantly increased in the case of HMGB1 stimulation from pulpitis compared with healthy pulps. (Figure 3A).

Frequency of Th17 cells in macrophages stimulated with HMGB1 and lymphocyte cells from pulpitis slide

The results of immunofluorescent staining showed that the number of Th17 cells from pulpitis was enhanced after HMGB1 stimulation *in vitro*, compared with healthy pulps ($P < 0.001$) (Figure 4).

Discussion

Pulpitis is inflammation of the dental pulp tissue. It is commonly associated with toothache. It uses receptors to recognize molecular patterns common to microbes to initiate bacterial killing (phagocytosis) (15, 16). As well

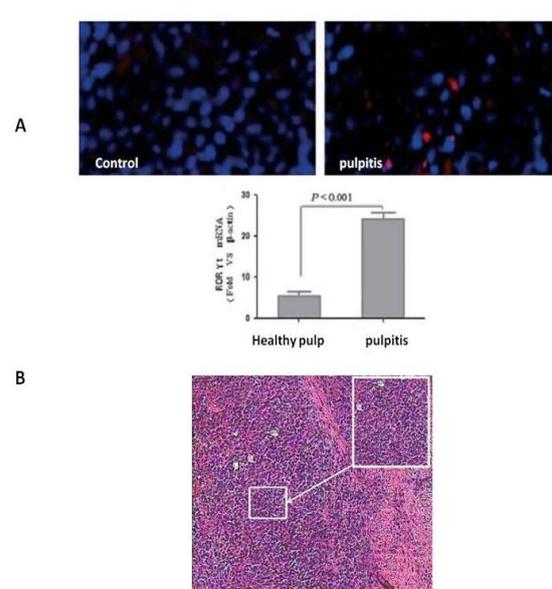


Figure 4. The results of immunofluorescent staining. The images in the upper half indicated the results of observation on immunofluorescent staining by fluorescence microscopy; the red cells represent positive staining cells (Th17 cells) and increased numbers could be found in CD4+ T cells from macrophages. The histogram of the lower half showed that the frequency of Th17 cells in CD4+ T cells from macrophages in pulpitis was increased ($P < 0.001$) compared with healthy pulp.

as lymphocytes, macrophages provide a defense against certain intracellular pathogens.

Activated macrophages are class II antigen-presenting cells (17). Activated macrophages also secrete many inflammatory mediators. Macrophages in pulps become activated after receiving two signals (18). The activating stimulus may include bacterial lipopolysaccharides, mural dipeptide, and other chemical mediators. Macrophages are professional phagocytes in innate immune responses (19). Activated macrophages can exclude pathogens in immune responses and remodeling and repair of tissue after inflammation (20).

Activation of cells by microbial components and endogenous molecules via TLRs results in the production of a variety of proinflammatory cytokines, chemokine, and destructive enzymes, some of which can characteristically be found in pulpitis (21).

TLR2 and TLR4 are types of TLR family members, expressed on macrophages, and the TLR2 signaling is thought to be essential for the inflammatory response and for immune disorders (22, 23). TLR2 and TLR4 mediated nuclear factor-kappa B (NF- κ B) signaling pathway contributes to the inflammatory process (24). Recently, pattern recognition receptors triggered by molecules of bacterial origin (the TLR family) were also shown to respond to HMGB1 (25, 26). HMGB1 is a dangerous protein that can bind to DNA (26). In the present study, recombinant human HMGB1 was used to stimulate CD16+ macrophages *in vitro* for understanding the effects of HMGB1 stimulation on TLR2 and TLR4 pathways.

It should also be noted that HMGB1 preparation should be defined as the content of decontaminants, most importantly LPS and DNA. Incubation of macrophages with LPS might result in increased release of HMGB1, and HMGB1 itself can stimulate macrophages and neutrophils to release proinflammatory cytokines, including IL-1b, TNF-a, IL-8, and other cytokines.

In this experiment, the pure HMGB1 obtained from the Invitrogen Company, USA was used. It has been demonstrated that IL-17 is mainly produced by Th17 cells, and NK, RAGE + T cells are also considered as IL-17-secreting cells (27, 28). As the same cytokine is produced by these different cells, IL-17 is a pivotal proinflammatory molecule in immune inflammation diseases. In this study, we found that Th17 cells were the main producers of IL-17, and macrophages could also produce IL-17 particularly in the case of HMGB1 stimulation. We hypothesized that the immune deregulation in pulpitis might potentially be due in part to increased frequency of Th17 cells, and the stimulation of HMGB1 secreted by activated cells or leaked by injured cells may induce Th17 cell differentiation via up-regulating TLR2 and TLR4 and proinflammatory cytokines such as IL-6. While testing this hypothesis, we detected the expression of TLR2 and TLR4 on CD16+ macrophages in pulpitis and its changes after HMGB1 stimulation *in vitro*. We also examined the levels of IL-17, IL-23, and IL-6 produced by HMGB1-stimulated macrophages.

We found enhanced expression of TLR2 and TLR4 on CD16+ macrophages stimulated with HMGB1, compared with controls of macrophages without HMGB1 stimulation. Furthermore, there were increased levels of IL-17, IL-23, and IL-6 in supernatants from cultured macrophages. NF- κ B, the downstream target of TLR2 and TLR4, also showed a marked elevation after macrophage stimulation by HMGB1. HMGB1 was structurally highly conserved and thus may also affect other HMGB1 receptors.

Further research confirmed that NF- κ B can be either directly or indirectly involved in the HMGB1-induced signal control process. Inhibition of NF- κ B can significantly decrease HMGB1 gene expression in animal tissues in endotoxic shock. Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway may also be involved in the expression of HMGB1 and inflammatory response signal regulation, although its mechanism is unclear (26).

To date, there has been little discussion on the role of HMGB1 in pulpitis; however, the interest has focused on the effect of HMGB1 in cancer, Rheumatology and autoimmunity disease (RA) (29). It is also difficult to ignore the role of HMGB1 in heart immunity disease, where it has been shown that it may modulate Th-17 cells, and its blockade suppresses Th-17 cells (30). In our previous study, we found that Th-17 cells and HMGB1 were increased in patients with RA and the immune deregulation in RA may be potentially due in part to increased frequency of Th-17 cells, and the stimulation of

HMGB1 secreted by activated cells or leaked by injured cells may induce Th-17 cell differentiation via up-regulated TLR2 and Th-17 cell-associated cytokines (31).

Conclusion

Our results are consistent with the conclusion that HMGB1 is an endogenous TLR2 ligand. This implies that the enhanced TLR2 and TLR4 pathways and Th17 cell polarization may be due to HMGB1 stimulation in pulpitis. In other words, HMGB1 can promote the differentiation of Th17 via up-regulation of TLR2 and TLR4 and IL-23 of CD16+ macrophages from pulpitis. At the same time, macrophages stimulated by HMGB1 may also secrete IL-17 and promote the development of inflammation. The present study suggests that the enhanced TLR2 and TLR4 pathways and Th17 cell polarization may stem from HMGB1 stimulation in pulpitis. Further investigations will also help to better understand the mechanism of pulpitis and design HMGB1-targeted therapies.

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Conflict of Interest

The authors declare no conflict of interest.

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