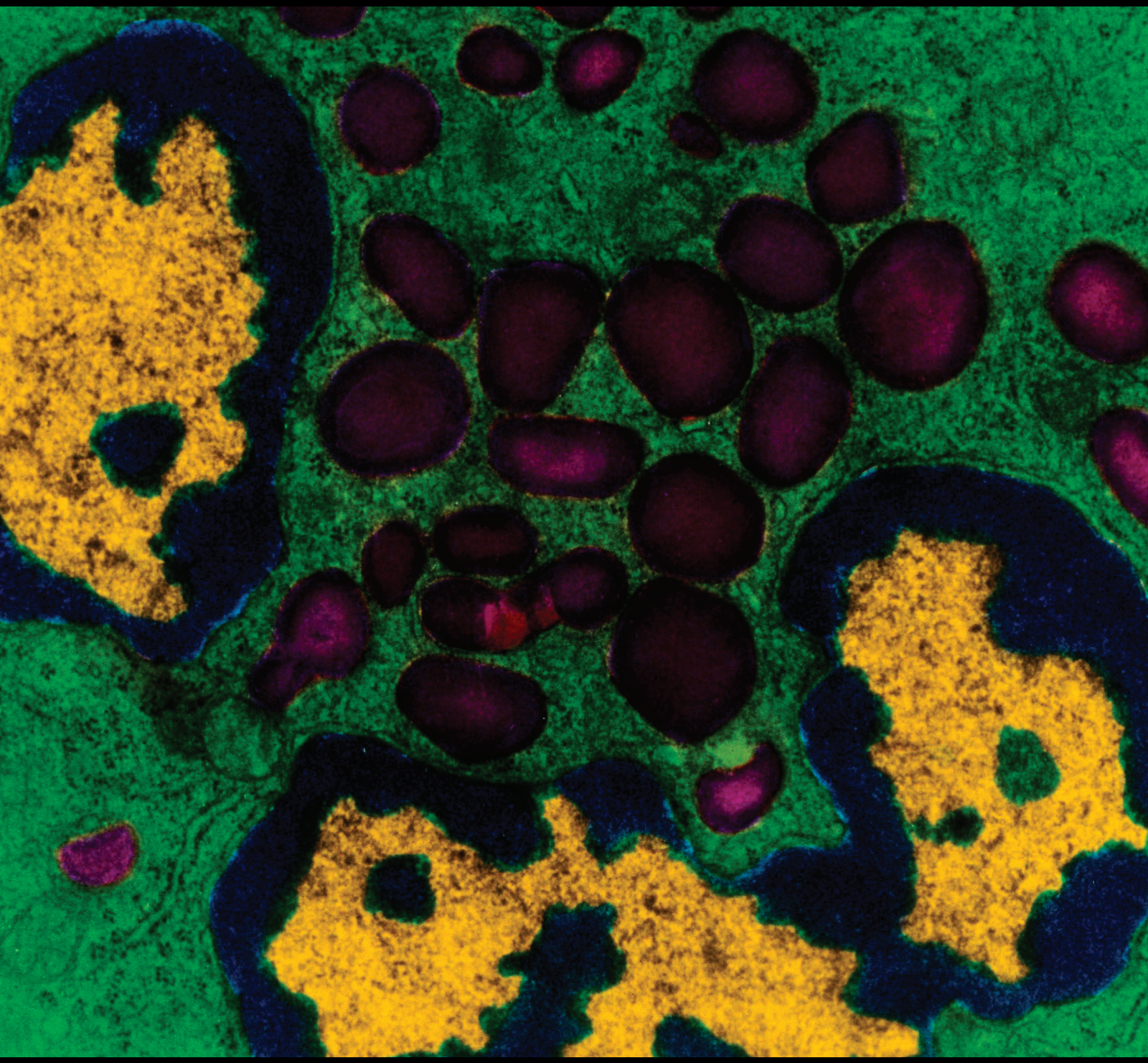
Mediators of Inflammation

# Inflammation of the Dental Pulp

Guest Editors: Sang Hyuk Park, Ling Ye, Robert M. Love, Jean-Christophe Farges, and Hiromichi Yumoto



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# Editorial Board

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*Editorial*

# Inflammation of the Dental Pulp

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The dental pulp is sensitive to external factors such as microbial infection from dental caries and/or mechanical/chemical irritations during dental procedures. Dental tissue behaves differently to the other connective tissues. It is unique in a way that its soft tissues (pulp and pulp-dentin complex) are enclosed within mineralized hard tissues (enamel, dentin, and cement), and its pulp is supplied by a rich neurovascular network that regulates various inflammatory mediators [1, 2]. Inflammatory signals may progress to rapid degeneration and necrosis, and such events could inflict very serious damage to tissues in the body [3, 4].

Factors that induce inflammation in the dental pulp and the root apex are as follows [1, 2, 5, 6]: the ingression of microorganisms through dental caries, crack or dentinal tubules of the teeth; chemical irritation from etching and/or bonding materials for adhesion of dental materials; mechanical irritation during preparation in restorative procedures; trauma from occlusion (TFO) or orthodontic movement of the teeth. Such factors may initiate the inflammatory cascades, which, in turn, further progress to pain and root resorption via neurogenic inflammation and hard tissue remodeling [7–9]. Therefore, a thorough understanding of the pulpal inflammatory process is essential in the development of proper dental procedures and immunotherapeutic agents.

This special issue is published with the intent of disseminating current knowledge and findings on inflammation in the dental pulp. This issue highlights the molecular mechanisms of inflammatory cascades, immunomodulatory effects of various substances, techniques used to study inflammation and regeneration of the dental pulp, and ultimately why an understanding of inflammatory process is important in the field of endodontics.

In this issue, J.-H. Jang et al. review pathogen recognition receptors (PRRs) for innate immunity in dental pulp. PRRs recognition of pathogen-associated molecular patterns (PAMPs) on pathogenic structure is important stage of initiating specific adaptive immunity. The paper reviews the various types of PRR families that include the Toll-like receptors (TLRs), the C-type lectin receptors (CLRs), the nucleotide-binding oligomerization domain-like receptors (NLRs), the retinoic acid-inducible gene-I-like receptors (RLRs), and the AIM2-like receptor (ALR). The authors suggest that immunomodulation via PRRs is crucial in the understanding of pathophysiology of pulp inflammation and also in the development of novel therapeutic targets in the pulp injury and/or infection.

J.-C. Farges et al. assess responses of the pulp tissues to bacterial infection at cellular and molecular levels. The pulp response is illustrated by mechanisms used by odontoblasts and specialised immune cells to resist pathogenic bacteria.

The cytokine IL-8 which are upregulated in response to bacterial component is particularly important, because it is involved in the recruitment and activation of neutrophils at the site of inflammation. This paper reports that the balance between the inflammatory response and the repair process is of paramount importance, as they dictate the rate of pulpal healing.

Y.-J. Ko et al. study the anti-inflammatory effect of human telomerase-derived peptide (GV1001) stimulated by lipopolysaccharide (LPS) from *P. gingivalis*. Confocal microscopy was used to analyse the intracellular distribution of GV1001, and real-time RT-PCR was performed to quantify the levels of TNF-$\alpha$ and IL-6 cytokines. The study reports that GV1001 was capable of entering the cell and caused downregulation of LPS-induced inflammatory cascades demonstrating its potential as a therapeutic agent in vital pulp therapy, regenerative endodontic fields, and tissue engineering.

S. Kim et al. review in vivo experiments with dental pulp stem cells to observe pulp-dentin complex regeneration. Most studies in this area have been conducted with mouse or dog models making their application in clinical environment unreliable. The authors suggest that where clinical trial of a study is impractical, orthotopic transplantation of dental pulp tissue cells (DPSCs) should be performed, as it would better predict impacts in human teeth. This paper claims that more emphasis in orthotopic transplantation in animal model is required to further expand our knowledge in pulp-dentin complex regeneration.

M. Goldberg et al. discuss the importance of inflammation in the pulp healing and regeneration. Despite its association with undesirable symptoms in pulpitis, inflammation should be understood as prerequisite of the pulp healing. The view is supported by inflammation-mediated proliferation of pulp cells and initiation of mineralisation. Inflammation may also be involved in the production of a reparative dentinal bridge that covers the pulp exposure.

D. Chavarria-Bolaños et al. conduct in vivo experiment to study sensory neuropeptides and endogenous opioids expression in human dental pulp with asymptomatic inflammation. Analysis of substance P (SP), calcitonin gene-related peptide (CGRP), $\beta$-endorphins ($\beta$-End), and methionine-enkephalin (Met-Enk) in patients' teeth with asymptomatic inflammation induced by orthodontic intrusion showed the significant increase in the level of SP and CGRP, both of which are frequently associated with pain of pulpal origin.

There is an ever growing interest in keeping teeth in a physiologically functional state in the event of injury and/or infection to the pulp, because no prosthesis can give as much stability and comfort as the normal teeth provide. Inflammatory process that takes place within a tooth is as unique as its structure compared to the other hard tissues in the body. Therefore, an understanding of inflammation of the pulp is a key to control pathophysiological status of the pulp/periapical tissues. This special issue includes reviews and research papers, which help to understand the process of inflammation in the context of dentistry and thereby expand our knowledge to potential therapeutic targets in pulp therapy.

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*Review Article*

# *In Vivo* Experiments with Dental Pulp Stem Cells for Pulp-Dentin Complex Regeneration

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In recent years, many studies have examined the pulp-dentin complex regeneration with DPSCs. While it is important to perform research on cells, scaffolds, and growth factors, it is also critical to develop animal models for preclinical trials. The development of a reproducible animal model of transplantation is essential for obtaining precise and accurate data *in vivo*. The efficacy of pulp regeneration should be assessed qualitatively and quantitatively using animal models. This review article sought to introduce *in vivo* experiments that have evaluated the potential of dental pulp stem cells for pulp-dentin complex regeneration. According to a review of various researches about DPSCs, the majority of studies have used subcutaneous mouse and dog teeth for animal models. There is no way to know which animal model will reproduce the clinical environment. If an animal model is developed which is easier to use and is useful in more situations than the currently popular models, it will be a substantial aid to studies examining pulp-dentin complex regeneration.

## 1. Introduction

Since Kakehashi et al. reported that bacteria caused pulpitis in their mouse experiment in 1965, endodontic treatment has developed with the goal of the complete removal of bacteria from the root canal [1]. It was believed that, once exposed to bacteria, the dental pulp would fall into a state of irreversible pulpitis that could never be restored to the normal state and that the progression of inflammation would result in necrosis. Therefore, the dental pulp tissues that are damaged due to bacterial invasion or dental trauma should be removed. The conventional root canal treatment focuses on three-dimensional mechanical preparation, disinfection, and the tight sealing of the root canal space to completely eliminate the dental pulp and bacteria in the root canal and prevent reinfection. However, this treatment is only a measure aimed at repair rather than true healing/regeneration. Thus, many studies have been conducted in the field of dental endodontics with the aim of pulp regeneration.

Two approaches have been undertaken for dental pulp-dentin complex regeneration. The first approach is the revascularization procedure. Many case reports have found clinical success following revascularization procedures, for example, no symptoms and no periapical lesions [2–4]. However, the histological observations from animal experiments have revealed that the tissues formed in the root canal do not reflect the regeneration of pulp-dentin but are rather formed of periodontal tissues, such as cementum, periodontal ligament, and bone [5, 6]. The revascularization procedure has its own clinical advantages in the treatment of immature teeth, but it does not result in pulp-dentin complex regeneration in the true sense.

The second approach for regenerating the dental pulp-dentin complex is the transplantation of mesenchymal stem cells into endodontically treated canals. In recent years, various stem cells have been isolated from the oral cavity. In 2000, Gronthos et al. isolated dental pulp stem cells (DPSCs) from human 3rd molars for the first time, and these

Table 1: Description of *in vivo* experiments with the application of DPSCs for pulp-dentin complex regeneration.

| Author | Animal | Ectopic/orthotopic | Source of stem cell |
|---|---|---|---|
| Shi et al., 2005 [17] | IC mice | Ectopic (subcutaneous) | hDPSCs, SHED, hPDLSCs |
| Okamoto et al., 2009 [18] | IC mice | Ectopic (subcutaneous) | hDPSCs |
| Alongi et al., 2010 [19] | IC mice | Ectopic (subcutaneous) | hDPSCs |
| Huang et al., 2010 [20] | IC mice | Ectopic (subcutaneous) | hDPSCs, SCAP |
| Wang et al., 2010 [21] | IC mice | Ectopic (subcutaneous) | hDPSCs |
| Galler et al., 2011 [22] | IC mice | Ectopic (subcutaneous) | hDPSCs, SHED |
| Lee et al., 2011 [23] | IC mice | Ectopic (subcutaneous) | hDPSCs |
| Choung et al., 2013 [25] | IC mice | Ectopic (subcutaneous) | hDPSCs |
| Yu et al., 2006 [26] | Rats | Ectopic (renal capsule) | hDPSCs |
| Yu et al., 2007 [27] | Rats | Ectopic (renal capsule) | hDPSCs, BMSSCs |
| Yu et al., 2008 [28] | Rats | Ectopic (renal capsule) | hDPSCs |
| Iohara et al., 2004 [29] | Dogs | Orthotopic (canine) | Porcine pulp cells |
| Iohara et al., 2009 [30] | Dogs | Orthotopic (canine) | SP cells from canine pulp |
| Iohara et al., 2011 [31] | Dogs | Orthotopic (incisor) | SP cells from canine pulp |
| Iohara et al., 2013 [32] | Dogs | Orthotopic (incisor) | MDPSCs |
| Iohara et al., 2014 [33] | Dogs | Orthotopic (incisor) | MDPSCs |
| Zhu et al., 2012 [34] | Dogs | Orthotopic (premolar) | cDPSCs |
| Kodonas et al., 2012 [35] | Mini pigs | Orthotopic (incisor root, jaw bone) | sDPSCs |

IC, immunocompromised; hDPSCs, human dental pulp stem cells; SHED, stem cells from human exfoliated deciduous teeth; hPDLSCs, human periodontal ligament stem cells; SCAP, stem cells from apical papilla; BMSSCs, bone marrow stromal stem cells; SP, side population; MDPSCs, DPSC subfraction based on their migratory response to granulocyte-colony stimulating factor; cDPSCs, canine dental pulp stem cells; sDPSCs, swine dental pulp stem cells.

DPSCs were characterized as highly proliferative cells with self-renewal multidifferentiation properties *in vitro* [7]. The dental pulp is vascularized and characterized by innervated loose connective tissue that contains heterogeneous cell populations [8]. Due to the complexity of pulpal tissues, no universally accepted protocols or cell types are currently available to assess pulp regeneration. However, a consensus exists regarding the importance of neural and vascular reinnervation for successful pulp regeneration [9]. DPSCs that exhibit pluripotent mesenchymal stem cell characteristics can be easily isolated from teeth [10]. Therefore, the strong angiogenic and neurogenic potentials of DPSCs have attracted much attention in the study of pulp regeneration [11–13].

Tissue engineering approaches are based on three central components: the living cells, scaffolds, and growth factors. Various types of stem cells, scaffolds, and growth factors have been researched and reported to function in pulp-dentin complex regeneration. Before applying these candidate substances in clinical trials, their biocompatibilities and treatment efficacies should be evaluated *in vivo* using animal models. In pulp-dentin complex regeneration research, the absence of animal models that adequately reflect pulp injury and pulpitis for investigation of the use of DPSCs has resulted in skipping preclinical testing. However, if the efficacy and safety of a treatment have not been validated in animal experiments, serious side effects can occur [14, 15]. Thus, the development of a reproducible transplantation animal model is essential for obtaining precise and accurate data *in vivo* [16]. The efficacy of pulp regeneration should be assessed qualitatively and quantitatively via the use of animal models.

This review article sought to introduce *in vivo* experiments that have evaluated the possibilities of dental pulp stem cells for pulp-dentin complex regeneration. This paper concentrates on the selection of *in vivo* and DPSC experiments that were identified in the Medline database via PubMed.

## 2. *In Vivo* Engineering in Dental Pulp Regeneration

*2.1. Ectopic Transplantation versus Orthotopic Transplantation.* The use of preclinical animal models is the most informative approach to obtaining clinically relevant data and verifying safety prior to human application. Animal model designs are categorized into ectopic transplantation and orthotopic transplantation. The word "ectopic" is from the Modern Latin from the Greek word *ektopos,* which means away from an original place or abnormal position. The word "orthotopic" is from the Greek word *orthos*, which means normal or usual position.

In pulp-dentin complex regeneration research, the ectopic implantation of stem cell involves transplantation of the cells into the outside of the tooth, for example, into subcutaneous tissue or the renal capsule. Orthotopic implantation involves the transplantation of the cells into the root canal space. Table 1 summarized current *in vivo* transplantation experiments with dental pulp stem cells.

*2.2. In Vivo DPSCs Experiments: Ectopic Transplantation.* It is not easy to apply the various types of cells, growth factors, and scaffolds that can be used controllably in *in vitro* experiments using animals. In early stages, *in vivo* experiments require large numbers of animals and easy access to the points of application. Ectopic transplantation not only

has the advantage of easy access but also has the advantages of being rapid and reproducible, requiring minimal labor, being relatively inexpensive, and producing easily quantifiable data [36]. Ectopic implantation is typically chosen for investigations of the characteristics of newly discovered stem cells and studies of the suitability of various scaffolds for dental tissue engineering [37]. Small animals, such as mice or rats, are preferred over large animals, such as monkeys, dogs, and pigs, due to costs, breeding management, and ethical issues.

Mice are the most commonly used species in ectopic transplantation experiments. Mice are relatively inexpensive and easy to handle and involve fewer ethical issues. Mouse anatomy is well known to researchers, and the mouse genome is 99% similar to the human genome [38]. Advances in genetic engineering technology have allowed for the generation of genetically modified mice. Severe combined immune deficiency (SCID) mice are naturally born without an immune system and can serve as models for allogeneic and xenogeneic DPSC transplantations.

Almost all of the ectopic DPSCs transplantation experiments have been performed in mouse subcutaneous tissue. Subcutaneous transplantation is technically easy compared with other sites and is less likely to result in the death of the animal. Many researchers have studied the properties of the mesenchymal stem cells derived from human dental tissue using mouse subcutaneous tissues. Shi et al. obtained dental pulp from impacted 3rd molars and digested tissues in collagenase/dispase to generate single-cell suspensions. Cultured DPSCs were cotransplanted with hydroxyapatite/tricalcium phosphate (HA/TCP) particles into the subcutaneous tissues of immunocompromised mice for 8 weeks. Consequently, donor-derived pulp-dentin-like tissues with distinct odontoblast layers lining the mineralized dentin matrix were generated [17]. Researchers have demonstrated the pulp-dentin complex regeneration capabilities of DPSCs in a variety of environments with mice since the initial *in vivo* and *in vitro* experiments identified the basic characteristics of DPSCs. Okamoto et al. cultured DPSCs with simvastatin for 7 days and transplanted DPSC-HA/TCP mixtures into the dorsal subcutaneous tissues of 10-week-old immunocompromised mice for 8 weeks. The DPSCs pretreated by high-concentration simvastatin formed significantly greater amounts of mineralized tissue than those treated with low concentrations and the controls [18]. Alongi et al. conducted an experiment with mice to examine whether DPSCs could be found in inflamed pulp as well as in normal pulp and if the DPSCs from inflamed pulp would retain their tissue regeneration potential following successful cultivation. These authors reported that DPSCs obtained from inflamed pulp also exhibited tissue regeneration capacities [19]. Huang et al. established an artificial human root canal model in which one end of the canal was opened to permit blood supply and the other end was sealed with MTA [20]. In research in which human root fragments were implanted into immunocompromised mice, DPSC cultures were observed to result in pulp-like tissues with well-established vascularities. This study provides the first evidence that pulp-like tissue can be generated by DPSCs in the empty root canal space and that odontoblast-like cells proliferate from DPSCs to produce dentin-like hard tissue on the dentinal walls. This research is significant in that it used a human root model that created an environment as similar as possible to that of orthotopic implantations to overcome the limitations of ectopic implantation. Additionally, research has been performed to assess the effectiveness of scaffolds and to identify the factors that influence the differentiation of DPSCs into odontoblasts [21–23, 25].

In addition to subcutaneous tissues, renal capsules are used in DPSC research. Yu et al. reported several *in vivo* studies using rat renal capsules [26–28]. The renal subcapsular space is a good site for cell transplantation due to its high graft uptake rate and abundant blood supply [39]. However, renal capsules are not used frequently because they are difficult to access compared with subcutaneous tissues.

*2.3. In Vivo DPSCs Experiments: Orthotopic Transplantation.* In dental pulp regeneration research, orthotopic transplantations involve implantation into the teeth, that is, the original anatomical site. When teeth are used in research, they can reflect the actual clinical environment better than ectopic sites such as subcutaneous tissues and renal capsules. However, it is not easy to implant a mixture of DPSCs and scaffolds into animal teeth. This procedure requires particularly advanced skills and techniques when using mice or rats, which have tooth diameters of only 2–5 mm. Therefore, larger animals, such as dogs and pigs, are often preferred over mice for this purpose [14].

Many dental experimental studies have been performed in dogs. Dogs are considered an ideal animal model for dental research due to the similarities in anatomy, growth patterns, and pathophysiologies with humans [40]. Dogs are diphyodonts with deciduous and permanent dentitions. The composition of the permanent dentition is three incisors, one canine, four premolars, and two molars in the maxilla and three incisors, one canine, four premolars, and three molars in the mandible. The beagle is one of the most commonly used dogs because this breed has a well-adjusted temperament and is easy to handle due to its proper size [41].

Almost all research into the orthotopic transplantation of DPSCs has been performed in dogs. Iohara et al. reported various studies about DPSC implantations in dog teeth [29–33]. In 2004, the efficacy of BMP2 on the differentiation of DPSCs into odontoblasts was examined in a canine amputated pulp model. A surgical exposure was made in the canine, and the amputation was performed. The upper incisor pulp was extracted and isolated, and the pulp cells were enzymatically separated. The pellet of the dog pulp cells was cultured and applied to the amputated root canal. Consequently, the transplantation of the BMP2-treated pulp cells into the amputated root canal stimulated reparative dentin formation [29]. Subsequently, Iohara et al. studied several factors that affect pulp regeneration using a surgically amputated canine pulp model. These authors researched the potential utility of a subfraction of a side population of cells, that is, $CD31^-/CD146^-$ and $CD105^+$ immunophenotype cells, from dog dental pulp for angiogenesis/vasculogenesis and pulp regeneration in a surgically amputated canine pulp model [30, 31]. These authors demonstrated successful

pulp-dentin complex regeneration in canine teeth. The same group also studied the effects of granulocyte colony-stimulating factor and host age on pulp regeneration [32, 33].

In addition to Iohara, other researchers have conducted research on pulp regeneration in dogs. Zhu et al. investigated the pulp regeneration potential of DPSCs and platelet-rich plasma (PRP) using the surgically amputated canine pulp model established by the Iohara group [34]. Contrary to the expectation that better results would be observed in a DPSC and PRP group than in a blood clot group, histologic examinations did not indicate any differences between the two groups. These findings opposed those from previous *in vitro* research that predicted that DPSCs plus PRP would play an important role in pulp-dentin complex regeneration because PRP contains many growth factors, and treatment with the appropriate concentration of PRP has been shown to enhance the proliferation and mineralization differentiation of DPSCs [24], which indicates that the results obtained in the laboratory may not lead to results in preclinical animal studies.

Several studies have employed mini pigs. Kodonas et al. demonstrated the regenerative capacity of swine DPSCs seeded in organic and synthetic scaffolds and implanted into the jaw bones of mini pigs [35]. These authors established a root implant model. The root implants were manufactured using the middle part of the roots of freshly extracted swine incisors, and the root canals were filled with scaffolds containing DPSCs and then implanted into the fresh postextraction sockets. This method is not truly orthotopic transplantation. However, it can be used as a valuable animal model for DPSC research in that it attempts to reproduce the clinical environment as much as possible.

*2.4. Other Orthotopic Transplantation Models.* Although not truly DPSC-based research, other efforts have been made to create an animal model to identify the elements that influence pulp regeneration [42, 43]. Torabinejad performed radiographic and histologic examinations with ferret cuspid teeth over 36–133 days after birth [43]. This author reported that ferret cuspid teeth exhibit anatomical, physiologic, histologic, and pathologic characteristics that are similar to those of human teeth. The ferret has four single-root teeth that are easily accessible for the performance of endodontic procedures and easy to evaluate radiographically and histologically. Torabinejad's model holds some significance as an animal model because ferrets are easier to handle and are cheaper than dogs and present fewer ethical issues than dogs, which are popular pets.

Other researchers have created orthotopic animal models with small animals instead of large animals, such as dogs, pigs, and ferrets. Simon et al. evaluate mice as an *in vivo* model for the study of pulpal healing in response to direct pulp capping [42]. These authors proposed the possibilities of orthotopic transplantation research with mouse teeth in preliminary studies despite the difficult accessibility and the small sizes of the teeth. Despite their many advantages as experiment animals, small animals, such as rodents, have been considered to be inappropriate for orthotopic transplantation animal models due to their small teeth [14, 43]. However, it became possible to apply DPSCs to the teeth of small animals and to perform histological and radiological observations of those teeth because of the use of microscopes, the developments in experimental instruments, and the introduction of scaffold-free approaches [42, 44]. Additional research on the development of rodent animal models will aid the development of new orthotopic models.

## 3. Conclusion

Recently, many studies have approached the pulp-dentin complex regeneration with DPSCs. While it is important to perform research on cells, scaffolds, and growth factors, it is also critical to develop animal models for preclinical trials. Although no human studies have yet been performed, research has been conducted with various types of animals and produced promising results.

According to a review of various studies of DPSCs, the majority of studies have used mouse subcutaneous tissues and dog teeth for animal models. There is no way to know which animal model better reproduces the clinical environment, but the results of an orthotopic transplantation experiment will be more reliable than those of ectopic transplantation experiments. The problem is that orthotopic transplantation research lags behind ectopic transplantation research in both quantity and quality. Sufficient orthotopic transplantation research should be conducted before clinical trials are initiated. If an animal model is developed which is easier to use and more versatile than the currently popular models (e.g., the canine pulp model), that model will be of substantial benefit to research on pulp-dentin complex regeneration.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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*Research Article*

# The Anti-Inflammatory Effect of Human Telomerase-Derived Peptide on *P. gingivalis* Lipopolysaccharide-Induced Inflammatory Cytokine Production and Its Mechanism in Human Dental Pulp Cells

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*Porphyromonas gingivalis* is considered with inducing pulpal inflammation and has lipopolysaccharide (LPS) as an inflammatory stimulator. GV1001 peptide has anticancer and anti-inflammation activity due to inhibiting activation of signaling molecules after penetration into the various types of cells. Therefore, this study examined inhibitory effect of GV1001 on dental pulp cells (hDPCs) stimulated by *P. gingivalis* LPS. The intracellular distribution of GV1001 was analyzed by confocal microscopy. Real-time RT-PCR was performed to determine the expression levels of TNF-$\alpha$ and IL-6 cytokines. The role of signaling by MAP kinases (ERK and p38) was explored using Western blot analysis. The effect of GV1001 peptide on hDPCs viability was measured by MTT assay. GV1001 was predominantly located in hDPC cytoplasm. The peptide inhibited *P. gingivalis* LPS-induced TNF-$\alpha$ and IL-6 production in hDPCs without significant cytotoxicity. Furthermore, GV1001 treatment markedly inhibited the phosphorylation of MAP kinases (ERK and p38) in LPS-stimulated hDPCs. GV1001 may prevent *P. gingivalis* LPS-induced inflammation of apical tissue. Also, these findings provide mechanistic insight into how GV1001 peptide causes anti-inflammatory actions in LPS-stimulated pulpitis without significantly affecting cell viability.

## 1. Introduction

Dentin pulp complex injuries are often induced by invasion of microorganisms and their components via dentinal tubules towards the pulp. Caries, cracks, fractures, and leakage from restorations provide pathways for microorganisms and their toxins to enter the pulp. Odontogenic infections are generally caused by polymicrobial and dominated by anaerobic bacteria [1]. The response of the pulpal irritation is inflammation and eventually pulp necrosis may occur. The inflammation can spread to the surrounding alveolar bone and cause periapical pathosis. In this process, bacterial lipopolysaccharides (LPSs) play a potential role in several responses to pulpal infection. Lipopolysaccharide (LPS) can induce the expression of proinflammatory cytokines and chemokines such as TNF-$\alpha$ and IL-6 and elicit the innate immune response in dental pulp cells (DPCs) [2].

Signaling pathways initiated by engagement of toll-like receptors (TLRs), such as TLR2 and TLR4, by bacterial products lead to enhanced transcription of genes responsible for the expression of cytokines, chemokines, adhesion molecules, and other mediators of the inflammatory response associated with bacterial infection. Of note, the activation of mitogen-activated protein kinases (MAPKs) is important in the production of inflammatory cytokines by LPS stimulation [3]. The MAPK family includes extracellular-signal-related

protein kinase (ERK), c-JUN N-terminal kinase/stress-activated protein kinases (JNK/SAP) and p38MAPK [4]. The MAPK signaling pathway is involved in various kinds of cellular processes including differentiation, development, proliferation, and survival, as well as cell death, depending on cell type and stimulus [5, 6]. Pulpal p38MAPK signaling is activated by LPS stimulation during the induction of local proinflammatory response [7–9].

Telomeres are specialized structures at the ends of chromosomes that have a role in protecting the chromosome ends from DNA repair and degradation [10]. Telomerase is a cellular reverse transcriptase (TERT, telomerase reverse transcriptase) which prevents premature telomere attrition and maintains normal length and function [11]. Human reverse transcriptase subunit of telomerase (hTERT) has become an attractive target for cancer vaccines due to it being expressed in 85–90% of human cancer tissues, whereas it is almost never expressed in normal tissues [12]. GV1001 peptide, which is a peptide corresponding to amino acids 611–626 of hTERT (EARPALLTSRLRFIPK), has been developed as a vaccine against various cancers and has been reported to have the ability to penetrate into various cells, including cancer cell lines and primary blood cells [13]. GV1001 was found to localize predominantly in the cytoplasm and could successfully deliver macromolecules such as proteins, DNA, and siRNA into cells [13]. Because of this novel pharmaceutical potential and cell-penetrating ability, as well as its own anticancer activity, GV1001 peptide is very promising for use in the medical field. Here, we observed that this peptide could also penetrate into human dental pulp stem cells and, furthermore, that it had a self-anti-inflammatory effect without affecting cell viability.

The purpose of this study was to evaluate the cell-penetrating function of GV1001 peptide in human dental pulp cells (hDPC) and to investigate the anti-inflammatory effect of GV1001 and its related mechanism in *P. gingivalis* LPS-induced inflammation through regression of inflammatory cytokine production.

## 2. Materials and Methods

*2.1. Synthesis of Peptides.* All of the peptides used in this study were synthesized by the Fmoc- (9-fluorenylmethoxycarbonyl-) based solid-phase method and characterized by Peptron Inc. (Daejeon, Korea). The purities of all peptides used in this study were greater than 95%, as determined by high-performance liquid chromatography.

*2.2. Cells and Cultivation.* This study was approved by the Seoul National University Dental Hospital Institutional Review Board. The impacted third molars of human adults were collected from 18- to 22-year-old patients after obtaining informed consent. The isolated dental pulp was cut into small pieces and digested in a solution of 3 mg mL$^{-1}$ type I collagenase and 4 mg mL$^{-1}$ dispase for 30–60 min at 37°C (Sigma Aldrich, St. Louis, MO, USA). Subsequently, the solution was filtered through a 70 mm cell strainer (Becton/Dickinson, Franklin Lakes, NJ, USA). The single-cell suspensions were seeded in 35 or 60 mm culture dishes and maintained in a culture media consisting of $\alpha$-minimum essential medium ($\alpha$-MEM; Invitrogen, Carlsbad, CA, USA) supplemented with 15% fetal bovine serum (FBS; Gibco-BRL, Life Technologies Inc., Gaithersburg, MD, USA), 0.292 mg mL$^{-1}$ glutamine (Invitrogen), 100 units mL$^{-1}$ penicillin G, 100 mg mL$^{-1}$ streptomycin, and 50 mg mL$^{-1}$ ascorbic acid (Sigma). The cells were incubated in a humidified atmosphere containing 5% $CO_2$ at 37°C and cells between 3 and 4 passages were used in the following experiments.

*2.3. Bacterial Species and LPS Extraction.* *Porphyromonas gingivalis* ATCC 33277 was purchased from the American Type Culture Collection (ATCC; Manassas, VA) and cultured with brain heart infusion broth (BHI; BD Bioscience, Sparks, MD, USA) supplemented with hemin (1 $\mu$g mL$^{-1}$) and vitamin K (0.2 $\mu$g mL$^{-1}$) in anaerobic conditions at 37°C. Lipopolysaccharide was extracted from *P. gingivalis* cultured according to the method described by Lee et al. [14]. *E. coli* LPS was purchased from Sigma (B55:05, St. Louis, MO, USA).

*2.4. Confocal Microscopy.* hDPCs were seeded and cultivated in 2-chamber glass slides (Nunc, Roskilde, Denmark) for 12 h. After washing with PBS, cells were incubated in serum-free OPTI-MEM for an hour. Fluorescein isothiocyanate-(FITC-) labeled 1, 10, and 50 $\mu$M of GV1001 peptides were added to cells and incubated for 2 h. The cells were fixed with 4% paraformaldehyde solution for 20 min at room temperature. Cells were stained with TO-PRO-3 Iodide 642/661 nm (Invitrogen, Grand Island, NY, USA) to visualize nuclei and were subjected to confocal microscopy. Colocalization of the peptides and nuclei was assessed using an FV1000 lager scanning confocal microscope (Olympus).

*2.5. Western Blot Analysis.* After addition of 0.75 $\mu$M–10 $\mu$M GV1001 to LPS-treated cells, the samples were prepared for electrophoresis and were separated using a 10% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel with a previously established buffer system [15]. After electrophoresis, the proteins were transferred onto a nitrocellulose membrane and blocked by Tris-buffered saline (TBST, 0.05% Tween-20) containing 5% nonfat dried milk. The membranes were then incubated with anti-p-ERK and anti-p-p38 (Cell Signaling Technology, Beverly, MA, USA) antibodies and subsequently washed with TBST. Antigen-antibody complexes were visualized using an enhanced chemiluminescent detection system (West-Zol, Intron, Seoul, Korea) by incubating membranes with goat anti-rabbit IgG or goat anti-mouse IgG antibody coupled to horseradish peroxidase (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), diluted at 1 : 2000. The blots were stripped and reprobed with an anti-$\beta$-actin polyclonal antibody to ensure that equal amounts of protein were used.

*2.6. Real-Time RT-PCR.* After treatment with 0.75–20 $\mu$g mL$^{-1}$ LPS for 2, 4, 6, and 8 h, total RNA was isolated from the cells using Trizol reagent (Life Technologies Inc., Gaithersburg, MD, USA) according to the manufacturer's suggested protocol and treated with DNase I (RNase-free,

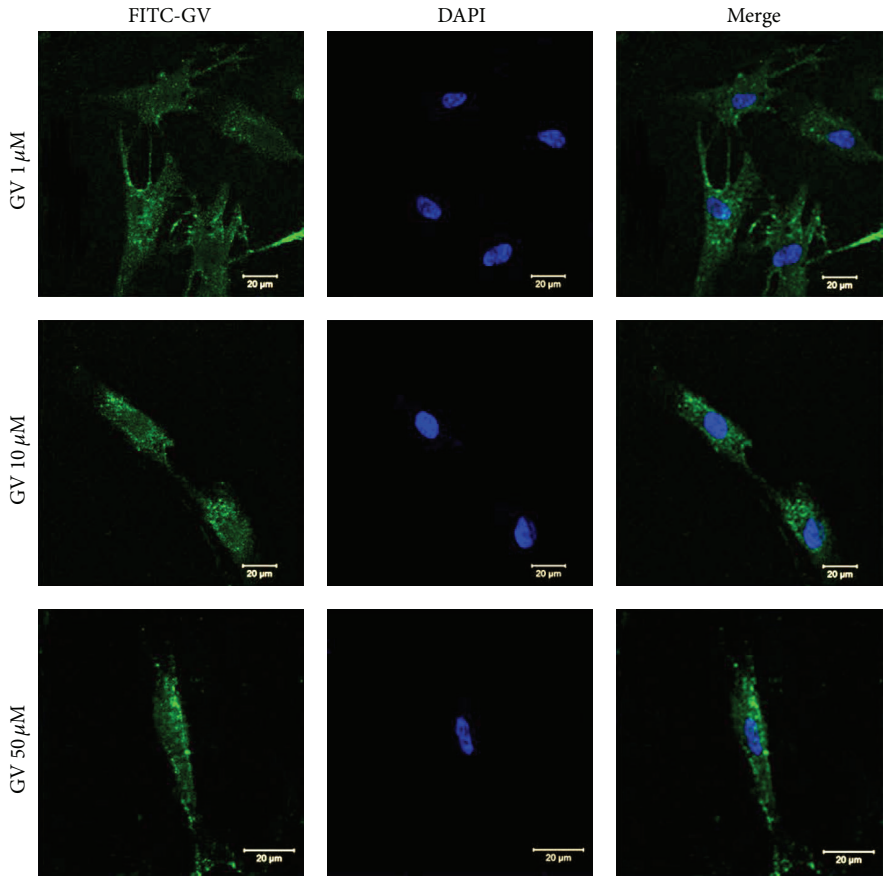



Figure 1: Internalization of various GV1001 concentration by hDPCs. 1, 10, and 50 $\mu$M GV1001 peptides labeled with FITC at the C-terminus (GV1001-F) were used to treat hDPCs as described in Section 2. Internalization of the peptides was analyzed by confocal microscopy.

RQ1; Promega, Madison, WI, USA). One microgram of total RNA was used as a template to create first-strand cDNA with oligo-dT priming using an Omniscript RT Kit (Qiagen Inc., Valencia, CA, USA). The quantitative real-time PCR analyses were performed using an ABI Prism 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with a SYBR Premix Ex Taq II kit (Takara, Otsu, Japan) and 35 cycles of PCR. The denaturing, annealing, and extension conditions of each PCR cycle were 95°C for 10 s, 60°C for 15 s, and 72°C for 10 s, respectively. The relative amount or fold change of the target gene was normalized relative to the level of the control (untreated cells). The following primer sequences were used in the real-time PCR reactions: 5′-CGA AAG TCA ACT CCA TCT GCC-3′ and 5′-GGC AAC TGG CTG GAA GTC TCT-3′ for IL-6 gene; 5′-CCA GGA GAA AGT CAG CCT CCT-3′ and 5′-TCA TAC CAG GGC TTG AGC TCA-3′ for TNF-$\alpha$ gene; 5′-GTG GTG GAC CTG ACC TGC-3′ and 5′-TGA GCT TGA CAA AGT GGT CG-3′ for GAPDH gene.

*2.7. Cytotoxicity Assay.* To evaluate the cytotoxicity of GV1001 on hDPCs, cells ($2 \times 10^5$/well) were treated with several concentrations of GV1001 (0, 1, 5, 10, and 50 $\mu$M/well) for 48 hours. The cells were incubated with 5.7 mol $L^{-1}$ of MTT solution for 4 h in a tissue-culture incubator. A 200-lL quantity of dimethyl sulfoxide solution was then added to the cell-culture wells, and the plates were shaken for 10 min at room temperature to dissolve the precipitated formazan crystals. The solution was centrifuged for 10 min, and the optical density of the supernatant was measured at wavelength of 540 nm using an ELISA plate reader (PowerWave X 340; BioTek Instruments, VT). 0.9% NaCl solution was used as a negative control. The MTT assay was performed three times.

*2.8. Statistical Analysis.* The data are expressed as the mean ± standard deviation (SD) of at least 3 separate experiments. Comparisons between 2 groups were analyzed using Mann-Whitney $U$ test. $P$ values less than 0.05 were considered statistically significant.

## 3. Results

*3.1. Internalization of GV1001 Peptide.* To confirm the cell-penetrating activity of GV1001, FITC was conjugated to the C-terminus of 1, 10, and 50 $\mu$M GV1001 peptides (GV1001-F) and subjected to an internalization assay using confocal microscopy. As shown in Figure 1, GV1001-F showed mostly cytoplasmic distribution in hDPCs in various levels of peptide concentration.

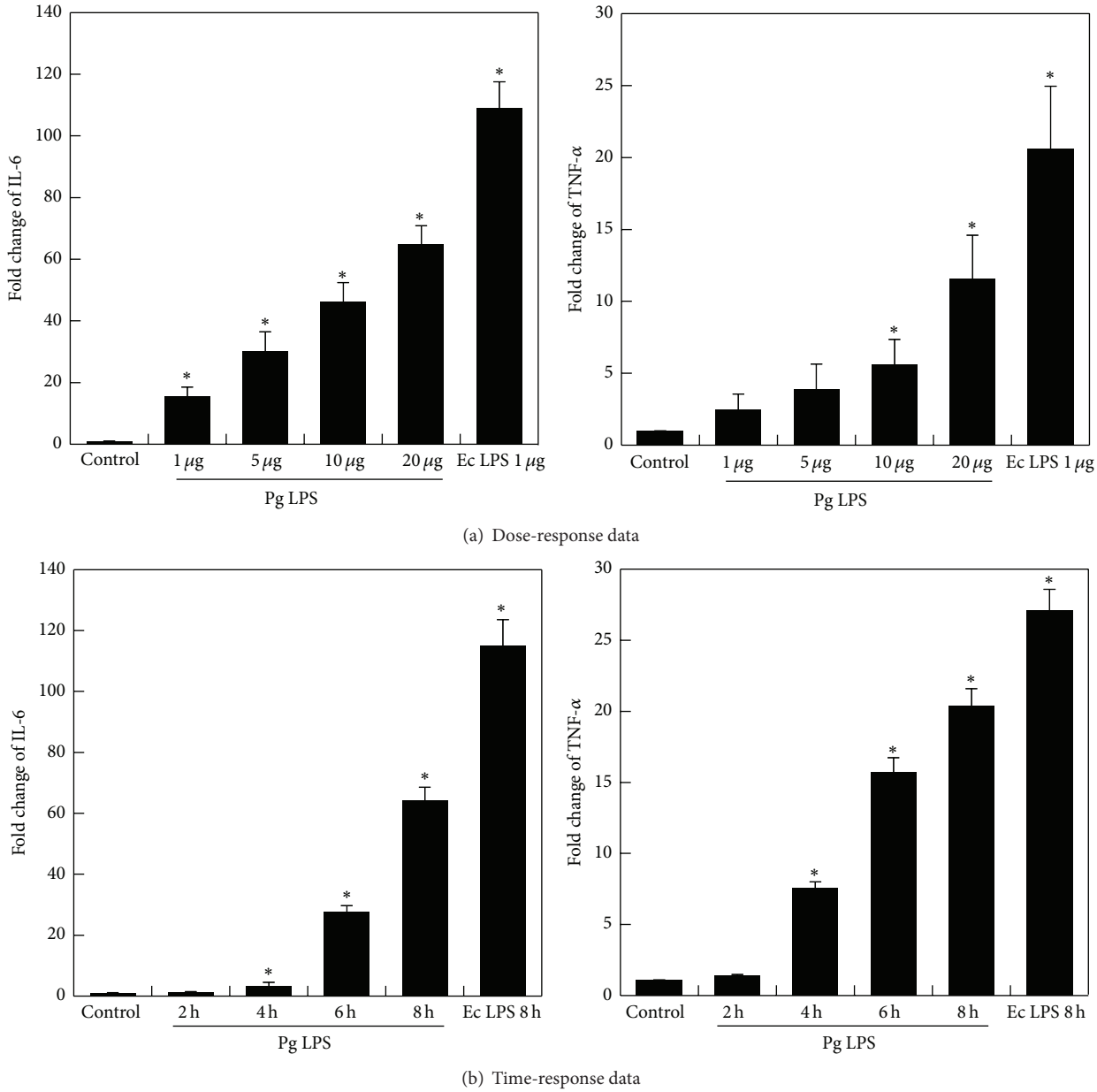
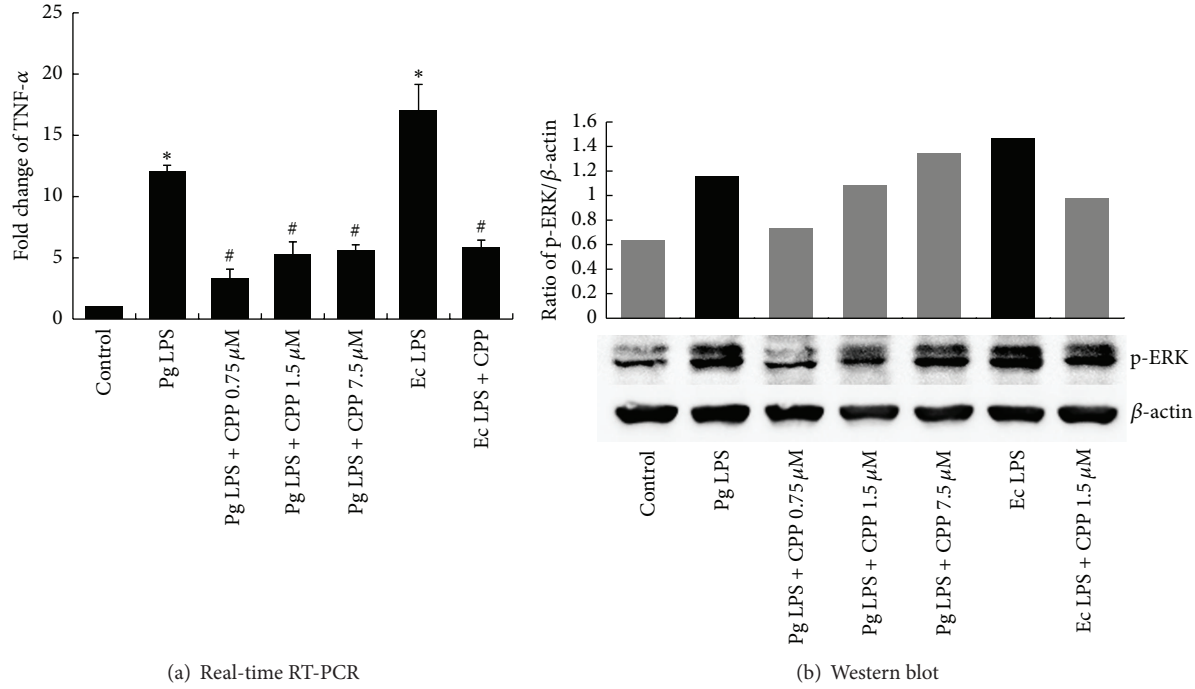



Figure 2: Effects of various doses of LPS and exposure times on the expression of TNF-$\alpha$ and IL-6 in hDPCs. hDPCs were serum-starved for 24 h and treated with indicated concentrations of *P. gingivalis* LPS (1, 5, 10, and 20 $\mu$g mL$^{-1}$) (a), for different times (2, 4, 6, and 8 hrs) (b). The levels of TNF-$\alpha$ and IL-6 mRNAs were determined by RT-PCR. Each value indicates the mean ± SEM of three independent experiments. $*$ indicates a significant difference ($P < 0.05$) relative to nontreated cells as control. *E. coli* LPS as positive control was treated 1 $\mu$g mL$^{-1}$.

*3.2. LPS of E. coli and P. gingivalis Induced Expression of Inflammatory Cytokines in hDPCs.* To verify the effects of various doses of LPS on inflammatory cytokine expression in hDPCs, the levels of TNF-$\alpha$ and IL-6 expression were evaluated using real-time PCR after exposing hDPC cells to LPS of *P. gingivalis* or 1 $\mu$g mL$^{-1}$ of *E. coli* at concentrations of 1, 5, 10, or 20 $\mu$g mL$^{-1}$ for 10 hrs. The results showed that IL-6 transcript was profoundly induced in the presence of 1, 5, 10, or 20 $\mu$g mL$^{-1}$ of *P. gingivalis* LPS or 1 $\mu$g mL$^{-1}$ of *E. coli* LPS ($P < 0.05$), and TNF-$\alpha$ transcript was profoundly induced in the presence of 10 or 20 $\mu$g mL$^{-1}$ of *P. gingivalis* or 1 $\mu$g mL$^{-1}$ of *E. coli* LPS ($P < 0.05$) (Figure 2(a)).

To evaluate the effects of LPS on inflammatory cytokine expression in hDPCs over a period of time, the levels of TNF-$\alpha$ and IL-6 expression were evaluated using real-time PCR after exposing hDPCs to 20 $\mu$g mL$^{-1}$ *of P. gingivalis* LPS or 1 $\mu$g mL$^{-1}$ of *E. coli* LPS for 2, 4, 6, or 8 hrs. Upregulation was found in hDPCs after 4 to 8 hrs of exposure to 20 $\mu$g mL$^{-1}$ of *P. gingivalis* LPS or 1 $\mu$g mL$^{-1}$ of *E. coli* LPS ($P < 0.05$) (Figure 2(b)). The expression of TNF-$\alpha$ and IL-6 in hDPCs




(a) Real-time RT-PCR (b) Western blot

Figure 3: Effects of GV1001 on LPS-induced TNF-$\alpha$ and p-ERK production in hDPCs. 0.75, 1.5, and 7.5 $\mu$M GV1001 were added to hDPSCs treated with 1 $\mu$g mL$^{-1}$ of *E. coli* LPS or 20 $\mu$g mL$^{-1}$ of *P. gingivalis* LPS for 10 hrs. RT-PCR and Western blot revealed downregulation of (a) TNF-$\alpha$ and (b) phosphorylated ERK, respectively, after exposure of cells incubated with GV1001 and 1 $\mu$g mL$^{-1}$ of *E. coli* LPS or 20 $\mu$g mL$^{-1}$ of *P. gingivalis* LPS. Each value indicates the mean ± SEM of three independent experiments. # indicates a significant difference ($P < 0.05$) relative to cells treated with LPS in the presence of GV1001.

were upregulated by LPS in a time- and dose-dependent manner.

*3.3. GV1001 Downregulated the Expression of TNF-α and IL-6 in LPS-Stimulated hDPCs.* We measured TNF-$\alpha$ and IL-6 expression after LPS stimulation to determine the anti-inflammatory activity of GV1001 peptide by real-time PCR. LPS-stimulated TNF-$\alpha$ and IL-6 production was significantly inhibited ($P < 0.05$) (Figures 3(a) and 4(a)).

*3.4. GV1001 Effectively Suppressed the Activation of ERK and p38MAPK by LPS Stimulation.* To investigate whether the anti-inflammatory effect of GV1001 is mediated by the suppression of ERK and p38MAPK activation, regulation of phosphorylated ERK and phosphorylated p38MAPK was evaluated by Western blot analysis. As shown in Figures 3(b) and 4(b), GV1001 decreased LPS-induced phosphorylation of ERK and p38MAPK.

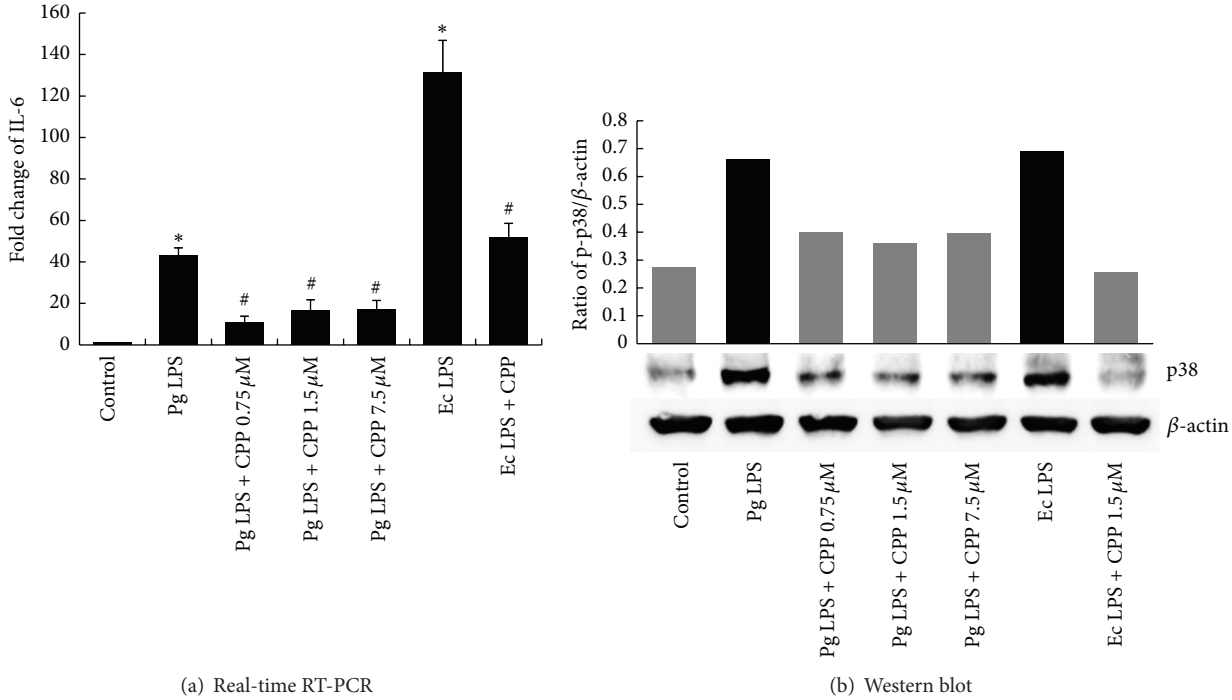
*3.5. GV1001 Has No Cytotoxic Effect on hDPCs.* hDPCs were exposed to GV1001, and cytotoxicity was tested by MTT assay. Incubating GV1001 peptide with hDPCs for 48 h did not affect cell viability in all concentrations tested (0–50 $\mu$M), which showed a pattern similar to that seen with 0.9% NaCl ($P < 0.05$) (Figure 5).

## 4. Discussion

Although endodontically treated teeth can maintain their function for a prolonged period of time, there are many advantages to maintaining pulp vitality. In immature permanent teeth with incomplete apical and dentinal wall development, reparative dentin formation is critical for further development of the teeth. Maintaining the vital pulp also has the benefit of reducing the occurrence of apical periodontitis by blocking bacterial infections [16]. Based on these advantages, it is preferable to maintain or renew pulp vitality rather than use current endodontic therapies. Recently, successful pulp regeneration and revascularization techniques have been developed and are gaining popularity.

*P. gingivalis* is an obligately anaerobic, Gram-negative bacterium that has been positively associated with destructive periodontal disease [17, 18]. It is one of the most pathogenic species among black-pigmented Gram-negative anaerobes [19]; it is frequently present in root canal infections and other odontogenic abscesses of patients not suffering from periodontal disease, which confirms its pathogenic role [20]. *P. gingivalis* may also play a role in symptomatic infections, such as acute apical abscess [21, 22]. Furthermore, there is a positive association between *Porphyromonas* spp. and pain, mechanical allodynia, swelling, and purulent exudates in root canals [22, 23].

Pathogens are generally detected by specific receptors, one of which is the toll-like receptor (TLR) family. TLR




(a) Real-time RT-PCR (b) Western blot

Figure 4: Effects of GV1001 on LPS-induced IL-6 and p-p38 expression in hDPCs. 0.75, 1.5, and 7.5 $\mu$M GV1001 was added to hDPCs treated with 1 $\mu$g mL$^{-1}$ of *E. coli* LPS or 20 $\mu$g mL$^{-1}$ of *P. gingivalis* LPS for 10 hrs. Real-time RT-PCR and Western blot revealed downregulation of (a) IL-6 and (b) phosphorylated p38, respectively, after exposure to 1 $\mu$g mL$^{-1}$ of *E. coli* LPS or 20 $\mu$g mL$^{-1}$ of *P. gingivalis* LPS incubated with GV1001 (0.75, 1.5 $\mu$M, and 7.5 $\mu$M). Each value indicates the mean ± SEM of three independent experiments. # indicates a significant difference ($P < 0.05$) relative to cells treated with LPS in the presence of GV1001.

stimulation initiates host defense through the activation of several intracellular signaling pathways, including activation of the mitogen-activated protein (MAP) kinases [24]. Exposure of cells to inflammatory stimuli, including LPS and proinflammatory cytokines, results in phosphorylation of p38MAPK [8].

Once the TLR is stimulated by a pathogen, proinflammatory cytokines and chemokines are produced by the odontoblast, resulting in recruitment and stimulation of immune effector cells and also direct bacterial killing [25]. Although these biological responses protect the host against invading pathogens, the inflammatory response also leads to host tissue damage. Hence, regulating the production of these cytokines is pivotal to protecting host tissue.

The extra-telomeric functions of hTERT are suggested regarding cellular proliferation, stem cell mobilization, and antiapoptotic, antiaging, and antioxidant effects through mitochondrial stabilization, transcriptional regulation [26, 27]. If hTERT peptide treatment that mimics the extra-telomeric functions of hTERT could be developed, it could be used in the treatment of pulpal diseases. In this study, the potential use of a small peptide of human origin to control pulpal inflammation as a therapeutic agent was investigated. The anti-inflammatory effect of GV1001 is achieved by modulating the suppression of the activation of ERK and p38 MAPK and the subsequent cytokine production induced by *P. gingivalis* LPS stimulation. *P. gingivalis* and *E. coli* LPSs induced TNF-$\alpha$ and IL-6 expression in hDPC in a time- and dose-dependent manner, while GV1001 peptide downregulated inflammatory cytokines, IL-6 and TNF-$\alpha$. hDPCs expressed TLR2 (data are not shown) and upregulated phosphorylated ERK and p38 MAPK in response to stimulation by LPS. From the similar suppression patterns of the two, it could be assumed that the downregulation of inflammatory IL-6 and TNF-$\alpha$ by GV1001 was dependent on p38 MAPK and ERK-signaling, respectively, thus indicating that MAPK signaling was required in downregulation of inflammatory cytokines in dental pulp stem cells by GV1001 peptide. As shown in Figure 3, higher dose of GV1001 exhibited a lower effect on TNF$\alpha$ induced by *P. gingivalis* LPS. One of the possible reasons is that GV1001 can partially aggregate in the high concentration in cell culture medium because cell culture medium does not include carrier protein. Thus, the lower concentration of GV1001 except aggregating GV1001 penetrates into the cells and reacts with signaling molecules related with inflammation.

In another previous study, GV1001 peptide was reported to have the ability to penetrate into various cells, including cancer cell lines and primary blood cells, without affecting cell viability [13]. GV1001 was predominantly located in the cytoplasm and was used to successfully deliver macromolecules such as proteins, DNA, and siRNA into cells [13]. These cell-penetrating peptides (CPPs) have become one of the most popular and efficient tools for delivering various molecules into cells owing to the fact that they have the ability to enter cells independently of a membrane receptor, and they

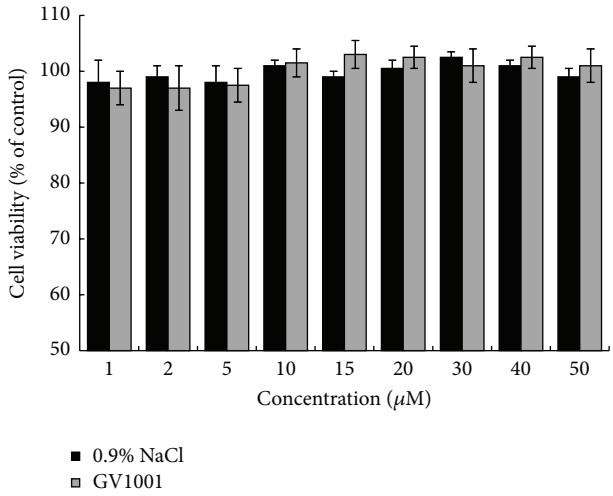



FIGURE 5: Effects of GV1001 on viability of hDPSCs. The cells ($2 \times 10^5$ cells/well) were treated with the indicated concentrations of GV1001 for 48 h. The cell viability was assessed by an MTT assay and the surviving cell values are shown as a percent of the control-treated cells (no addition of GV1001); 0.9% NaCl solution was used as a negative control. Each value indicates the mean ± STDEV of three independent experiments.

show no cell-type specificity [28]. This study is significant in that it is the first to demonstrate GV1001 peptide as a CPP of human origin with a self-anti-inflammatory effect and without affecting cell viability.

The use of GV1001 peptide can be a potential therapeutic approach for treating pulpal inflammation and the peptide can also be used as an intracellular delivery tool for bioactive molecules. Using GV1001 peptide as a pulp-capping agent on reversibly inflamed pulp or an alternative to antibiotics in regeneration therapy may be effective. Conjugating growth factors such as TGF-$\beta$s and BMPs with GV1001 can facilitate induction of hDPCs to differentiate effectively into odontoblast-like cells. Moreover, gene therapy can be implemented using GV1001 to fuse a growth/differentiation factor for application in vital pulp therapy, regenerative endodontic fields and tissue engineering. The results from this study may support further research on the GV1001 peptide and its various clinical applications.

## 5. Conclusion

GV1001 has the ability to penetrate into the cell. The down-regulation of LPS-induced TNF-$\alpha$ and IL-6 expression was mediated through ERK and p38 MAP kinase pathways. These findings provide mechanistic insight into the ability of GV1001 peptide to cause anti-inflammatory actions in LPS-stimulated pulpal inflammation without significantly affecting cell viability.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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*Review Article*

# An Overview of Pathogen Recognition Receptors for Innate Immunity in Dental Pulp

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Pathogen recognition receptors (PRRs) are a class of germ line-encoded receptors that recognize pathogen-associated molecular patterns (PAMPs). The activation of PRRs is crucial for the initiation of innate immunity, which plays a key role in first-line defense until more specific adaptive immunity is developed. PRRs differ in the signaling cascades and host responses activated by their engagement and in their tissue distribution. Currently identified PRR families are the Toll-like receptors (TLRs), the C-type lectin receptors (CLRs), the nucleotide-binding oligomerization domain-like receptors (NLRs), the retinoic acid-inducible gene-I-like receptors (RLRs), and the AIM2-like receptor (ALR). The environment of the dental pulp is substantially different from that of other tissues of the body. Dental pulp resides in a low compliance root canal system that limits the expansion of pulpal tissues during inflammatory processes. An understanding of the PRRs in dental pulp is important for immunomodulation and hence for developing therapeutic targets in the field of endodontics. Here we comprehensively review recent finding on the PRRs and the mechanisms by which innate immunity is activated. We focus on the PRRs expressed on dental pulp and periapical tissues and their role in dental pulp inflammation.

## 1. Introduction

The innate immune response is the first line of defense against infectious diseases and tissue damage. Macrophages and dendritic cells (DCs), as well as some nonprofessional cells such as epithelial cells, endothelial cells, and fibroblasts, play major roles in pathogen recognition during the innate immune response [1]. Cells of the host recognize structures called pathogen-associated molecular patterns (PAMPs) via germ line-encoded pattern recognition receptors (PRRs) present in their extracellular milieu and endosomal compartments [2]. Currently, PRR families are divided into transmembrane receptors and those that reside in intracellular compartments. The former include the Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), and the latter, the nucleotide-binding oligomerization domain- (NOD-) like receptors (NLRs), retinoic acid-inducible gene- (RIG-) I-like receptors (RLRs), and AIM2-like receptor (ALR) [1, 3, 4]. PAMP recognition by PRRs is influenced by both the responding cell and the invading microorganism. The signal transduction pathways that are activated via PRRs converge on a common set of signaling modules including nuclear factor- (NF-) $\kappa$B, activator protein-1 (AP-1), and mitogen-activated protein kinase (MAPK). These modules drive the production of

proinflammatory cytokines/chemokines such as interleukin- (IL-) 1, tumor necrosis factor- (TNF-) $\alpha$, and IL-6 [1, 2, 5]. Cytokines are multifunctional proteins that regulate osteoclast formation and hence bone resorption, modify vascular endothelial permeability, and recruit immune cells to inflamed tissue [2, 6].

Over the past decade there have been rapid advances in understanding innate immunity, particularly with regard to the mechanisms by which microbes are recognized and how the signaling molecules respond to them. Accumulating evidence of a relationship between bacterial recognition systems and oral disease has focused attention on the role of dental pulp tissues and their associated pathogens in innate immunity. In this review, we comprehensively review recent finding on the PRRs and the mechanisms by which innate immunity is activated. We will describe recent findings concerning the receptors for innate immunity in dental pulp.

## 2. Dental Pathogens and Innate Immunity

Teeth have unique structural features not found in any other tissue of the body. The hard tissues, enamel and dentin, make up the rigid external surface of the tooth, while its internal milieu is composed of soft tissue called "pulp." The pulp responds to external pathologic stimuli such as bacterial ingress and trauma, as well as thermal and chemical irritation during dental operations, all of which may induce inflammation [7, 8]. Pulp resides in a low compliance root canal system that limits the expansion of inflamed pulpal tissue that is invaded by inflammatory cells and whose blood vessels dilate [9, 10].

In the interface layer between dentin and pulp, there is a thin border which consists of odontoblasts and cells in a subodontoblastic layer [11]. Odontoblasts, the most highly differentiated cells of the pulp, are postmitotic neural crest-derived cells whose primary function is to elaborate dentin [12]. In response to irritation by cariogenic bacteria, odontoblasts produce tertiary dentin [13]. This has been classified as either reactionary or reparative, to distinguish between the events taking place in response to weaker versus stronger stimuli, and results from upregulation of the secretory activity of existing odontoblasts [12]. If the pulp is exposed, odontoblasts in the dentin pulp can no longer perform reparative processes. In the pulp, fibroblasts are the most numerous connective tissue cells, and they synthesize and maintain the connective tissue matrix [11]. Cariogenic bacteria trigger inflammatory and immune events in the underlying dental pulp via diffusion of their by-product into dentin tubules. If the bacteria are not eliminated, lesions progress to pulp inflammation and are followed by infection of the root canal system and periapical tissues and eventually by periapical disease [13].

Dental pathogens gain access to the dental pulp through the carious process and/or iatrogenic damage from dental treatments including cavity preparation and the use of cytotoxic dental materials. Dental caries harbour a wide range of bacteria, viruses, fungi, and protozoa within the mineralized tissues and canals of the root [14, 15]. When enamel structure is destructed, the dentin exposed to the oral microflora is degraded by Gram-positive bacteria, such as streptococci, lactobacilli, and actinomyces. Once bacterial infection due to dental caries progresses to the dentin-pulp interface, microflora is changed drastically. It is characterized by a reduction of Gram-positive aerobic bacteria with an increase of Gram-negative anaerobic bacteria, and initial pulpal immune response is activated [16–18]. It releases various bacterial toxins such as lipopolysaccharide (LPS), lipoteichoic acid (LTA), and some noxious metabolic by-products that will induce pulpal and periapical inflammatory reaction, followed by the results in irreversible pulpitis, pulp necrosis, and periapical disease [19–21].

It has been estimated that the human oral cavity is colonized by over 700 different species of bacteria [22]. The surface of the tooth accumulates bacteria in biofilms. The main bacterial species include streptococci (such as *Streptococcus mutans*) and *Actinomyces* spp. [23, 24]. The gingival crevices contain Gram-negative anaerobes such as *Porphyromonas gingivalis*, many of which are believed to be important in the development of periodontal disease [22, 24]. *Candida albicans* is the most common fungus present in the oral cavity, especially in the root canals [24, 25]. Protozoa, such as *Entamoeba gingivalis,* and viruses, including herpes virus and cytomegalovirus, are often present in the mouth [25]. Bacteraemia, endocarditis, atherosclerosis, and other cardiovascular diseases have been linked to oral pathogens that gain systemic access [26].

The innate immune system is the first line of pulp defense, triggered by pathogen recognition in a cell-autonomous manner [27]. The inflammatory process is mediated by PRRs which are expressed by various immune and nonimmune cells [2]. Innate immunity depends on the release of local mediators and phagocytic cells such as macrophages, monocytes, neutrophils, and DCs, whereas adaptive immunity uses antigen-specific T and B cells [28]. Phagocytic cells form an important part of the innate immune response. These cells directly remove pathogens that they encounter by phagocytosis but also release inflammatory cytokines and chemokines, which recruit other immune cells to the site of infection [29]. The expression of PRRs on host cells allows them to recognize specific pathogens, hence conferring a degree of specificity to the innate immune system. The DCs also express PRRs and act as cellular messenger by binding antigens and migrating to the lymph nodes where they activate the adaptive immune system [30]. The activation of PRRs can cause apoptosis and inflammation as well as stimulating adaptive immunity [1, 2, 31].

## 3. Pathogen Recognition Receptors

The defense mechanisms of the dental pulp comprise both innate and adaptive immunity. A critical first step in initiating an innate immune response to infection is the sensing of the pathogens by host cells. This is mediated by the recognition of specific microbial molecules by a limited array of dedicated host receptors. The microbial ligands, corresponding to essential components of the pathogen, are PAMPs and their cognate PRRs. As we described previously, PRRs are classified into five main families: TLRs and CLRs, transmembrane

proteins found in the plasma membrane, and RLR, ALR, and the NLR proteins located in intracellular compartment [3]. Here, we describe each of the PRR families and review recent findings on PRRs.

*3.1. Toll-Like Receptors.* Toll-like receptors (TLRs) are a class of proteins that play a key role in the innate immune system and received their name from their similarity to the protein encoded by the *toll* gene in *Drosophila* [56]. Drosophila Toll is involved in both embryonic development and the immune response to fungi [56, 57]. TLRs are a family of receptors with conserved architecture consisting of leucine-rich repeat- (LRR-) containing ectodomains and intracellular Toll-interleukin-1 receptor (TIR) signaling domains. The TLR ectodomains contain numerous LRRs, each repeat consisting of a 24-residue motif [1, 58].

The TLRs include TLR1–TLR10 and TLR11–TLR13, though the latter three are not found in humans. There are 10 TLR family members, TLR1–TLR10, in humans and 12, TLR1–TLR9 and TLR11–TLR13, in mice [59]. TLRs are able to recognize a variety of PAMPs including lipoproteins and di- and triacyl lipopeptides (TLR2/1 and TLR2/6), peptidoglycan, lipoteichoic acid, fungal zymosan (TLR2), double-stranded RNA (TLR3), flagellin (TLR5), unmethylated CpG DNA (TLR9), and a variety of synthetic molecules such as imidazoquinolines and guanosine analogues (TLR8). These molecules are recognized by individual TLRs in combination with coreceptors, or by TLR heterodimers [59]. Different TLRs appear to play crucial roles in the initiation of immune responses by recognizing different PAMPs. Odontoblasts constitutively express the PRRs TLR1–TLR6 and TLR9 genes [60].

TLR signaling is triggered by the ectodomain-mediated dimerization of TLRs. Its signaling involves two distinct signaling pathways: the myeloid differentiation factor 88- (MyD88-) dependent and TIR (Toll-interleukin receptor) domain containing adapter-inducing interferon- (IFN-) $\beta$- (TRIF-) dependent pathway. Those signaling pathways lead to activation of NF-$\kappa$B protein, which is a cytoplasmic transcription factor that initiates transcription of a wide range of genes involved in the inflammatory response including cytokines, chemokines, and immunoreceptors [58]. MyD88 is utilized by all TLRs with the exception of TLR3 and drives NF-$\kappa$B and MAPK activation to control inflammation [61]. TLR3 and TLR4 utilize the TRIF-dependent pathway, which is triggered by dsRNA and LPS, respectively [53, 62]. TRIF is also known as TIR domain containing adapter molecule (TICAM) 1, and it selectively recruited to their respective TLRs, eliciting appropriate responses depending on the type of PAMP [2].

TLR2 has also been designated CD 282. It is a surface membrane receptor that recognizes foreign substances and signals to cells of the immune system [63]. LTA stimulates the activation of odontoblasts, which is followed by expression of its receptor, TLR2 [32]. Murine pulp fibroblasts and odontoblasts have been shown to express TLR2 [39]. TLR2 is involved in the recognition and development of immunological responses against Gram-positive bacteria; it has a major role in the detection of peptidoglycan, lipoprotein, and LTA [39]. When TLR2 interacts with LTA, TLR2 gene expression in the cell membrane increases, NF-$\kappa$B translocates to the nucleus inducing the production of chemokines (via the chemokine genes *CCL2* and *CXCL8*), and immature DCs are recruited by upregulation of NOD2 expression [19, 35]. TLR2 is closely related to TLR1 and TLR6 with which it forms heterodimers that recognize bacterial lipoproteins and lipopeptides [34, 64]. TLR2 and TLR6 are required for responses to the diacyl lipoprotein from *Mycoplasma fermentans* [65]. TLR2 is expressed by neutrophils, mast cells, monocytes and macrophages, T cells, and B cells [45]. Macrophages and lymphocytes are the most prevalent cells in periapical infiltrates and produce IL-1, TNF, nitric oxide (NO), and reactive oxygen species (ROS). During the development of periapical lesions, macrophages and other innate immune response cells recognize bacterial constituents via specific receptors and initiate the inflammatory cascade [35, 45].

TLR4 is crucial for the detection of LPS, which is present in the cell wall of Gram-negative bacteria [66]. TLR4 is also expressed in the odontoblastic layer and pulp tissues [33]. Upon activation, TLR4 induces the production of proinflammatory cytokines, and cytokine expression is associated with bone resorption and tissue breakdown in endodontic periapical lesions [34, 35]. Mutoh et al. investigated the expression of TLR2 and TLR4 in inflamed dental pulp. They showed that TLR2 was strongly expressed on macrophages and DCs. TLR4-positive cells were also detected in the pulp, but the number of cells expressing it was much lower than in the case of TLR 2 [34].

The TLR5 ligand is flagellin, the major component of the bacterial flagellum and the structure responsible for motility in a wide variety of bacterial species [67]. A range of flagellated bacteria, but not aflagellate strains, activate TLR5 [68]. Interestingly, certain species of bacteria including *Campylobacter jejuni*, *Helicobacter pylori*, and *Bartonella bacilliformis* possess a divergent flagellin, which is not recognized by TLR5 due to amino acid mutations at residues 89–96 [68]. These amino acids correspond to a region previously defined as important for TLR5 activation. They are critical for flagellar filament formation and motility in other species, which explains their extensive conservation. Compensatory mutations in other regions of the protein are able to restore motility in these divergent species [68].

TLR3, TLR7, TLR8, and TLR9 are different from other TLRs in a view point in which they are not expressed in surface but localized on cytoplasmic vesicles such as endosomes. They are involved in the recognition of nucleic acids, with TLR3, TLR7, and TLR8 detecting double- and single-stranded RNA, respectively, and TLR9 detecting unmethylated CpG DNA [63]. In recognizing double-stranded RNA, TLR3 acts as a viral receptor, as dsRNA is present in certain viruses. Examination of the crystal structure of the human TLR3 ectodomain, in combination with mutational analysis, has identified two highly conserved residues critical for ligand binding and TLR3 activation within LRR 20 of the ectodomain [63].

TLR7 and TLR8 are structurally highly conserved proteins that interact with some of the same ligands. They are

Table 1: Summary of TLRs and NLRs in human innate immunity.

| Family | PRR | Location | Ligand (ligand location) | Unique features |
|---|---|---|---|---|
| Toll-like receptors (TLRs) | TLR1 | Cell surface | Triacyl lipopeptides (bacterial lipoprotein) | Formation of heterophilic dimers with TLR2 |
| | TLR2 | Cell surface | Di-/triacyl lipopeptides<br>Multiple lipoproteins<br>Lipoteichoic acid<br>Zymosan (fungi) | Formation of heterophilic dimers with TLR1 and TLR6 |
| | TLR3 | Endosome | dsRNA (virus) | dsRNA interacting with the N-terminal and C-terminal sites on the lateral side of convex surface of TLR3 |
| | TLR4 | Cell surface | LPS (Gram-negative bacteria) | Recognition of LPS together with myeloid differentiation factor 2 |
| | TLR5 | Cell surface | Flagellin | Activation of lung epithelial cells to induce inflammatory cytokine |
| | TLR6 | Cell surface | Triacyl lipopeptides (bacterial lipoprotein) | Formation of heterophilic dimers with TLR2 |
| | TLR7 and TLR8 | Endosome | ssRNA (virus) | Recognition of synthetic compound imidazoquinoline |
| | TLR9 | Endosome | Unmethylated CpG DNA | Involvement in the pathogenesis of autoimmune disorders through recognition of the chromatin structure |
| NOD-like receptors (NLRs) | NOD1 | Cytoplasm | Peptidoglycan (Gram-negative bacteria) | Recognition of intracellular bacterial cell products |
| | NOD2 | Cytoplasm | Peptidoglycan (Gram-positive bacteria) | |
| | NALP3 | Endosome | PAMPs, virulence factor<br>DAMPs | Response to multiple stimuli via forming a NALP3 inflammasome |

LPS: lipopolysaccharide; NOD: nucleotide-binding oligomerization domain; NALP3: NACHT [neuronal apoptosis inhibitory protein (NAIP), CIITA, HET-E, and TP-1] domain, LRR (leucine-rich repeat) domain, and PYD (pyrin domain) containing protein 3; PAMPs: pathogen-associated molecular patterns; DAMPs: danger-associated molecular patterns.

predicted to recognize the nucleic acid structures of viruses [69]. TLR7 is required for the normal IFN-$\alpha$ response to influenza in murine DCs [63]. In contrast, human TLR8 responds to these oligonucleotides independently of TLR7, pointing to species-specific differences between human and mouse. Binding of the single-stranded RNA virus, vesicular stomatitis virus (VSV), to mouse TLR9 also induces secretion of IFN-$\alpha$. This process requires the acidification of lysosomes and is inhibited by chloroquine, implying compartmentalization of the TLR7 response, possibly as a way of distinguishing self-RNA from non-self-RNA [69]. A number of other compounds activate TLR7, including various synthetic analogues of guanine, such as imiquimod, resiquimod, and loxoribine. Most are specific to TLR7, although resiquimod is also able to activate TLR8 [70]. Table 1 presents the overview of the TLRs and their PAMPs in human innate immunity.

*3.2. C-Type Lectin Receptors.* CLRs possess a transmembrane PRR with a carbohydrate-binding domain. CLRs recognize carbohydrates on pathogens and are mainly expressed by monocytes, macrophages, and DCs [3]. Pathogen recognition by CLRs leads to pathogen internalization and degradation and subsequent antigen presentation. CLRs recognize mannose, fucose, and glucan carbohydrate structures present in bacterial, viral, and fungal components. They are crucial for controlling both innate and adaptive immune responses. Some CLRs induce signaling pathways that modulate TLR-induced gene expression [71]. Mincle, a C-type lectin, detects infection by fungi; and it is sensed on both monocytes and neutrophils as well as on macrophages differentiated *in vitro* [72]. Inflammatory responses are crucial in innate immunity against infectious disease, but the factors that determine the dominant cellular component have not been identified. The association between these patterns and phagocyte function is currently being investigated.

*3.3. Nod-Like Receptors.* The nucleotide-binding oligomerization domain receptors, in short NLRs, are intracellular sensors of PAMPs that enter the cell via phagocytosis or pores and of danger-associated molecular patterns (DAMPs) that are associated with cell stress. They are pattern recognition receptors and play key roles in regulating the innate immune response. NLRs can cooperate with TLRs and regulate inflammatory and apoptotic responses. They are found in lymphocytes, macrophages, and DCs and also in nonimmune cells, for example, in epithelia. NLRs are characterized by their cytoplasmic location and the possession of a nucleotide-binding domain (NBD), which is also emerging as an important component of the innate immune response [4]. NLRs constitute a large family of intracellular PRRs, several of which—such as NOD1, NOD2, and NALP3 (which are characterized by NACHT [neuronal apoptosis inhibitory protein

(NAIP), CIITA, HET-E, and TP-1] domain, LRR (leucine-rich repeat) domain, and PYD (pyrin domain) containing protein 3) [2]. NALP3 is also known as NLRP3 (NLR family which has pyrin domain containing protein 3).

NOD1 and NOD2 recognize peptidoglycan components common to both Gram-positive and Gram-negative bacteria. Both proteins drive activation of MAPK and NF-$\kappa$B pathways, leading to proinflammatory cytokine production [2, 73]. Girardin et al. proved that human NOD1 specifically recognized a unique muropeptide motif found in Gram-negative bacterial peptidoglycan, resulting in activation of the NF-$\kappa$B responses [73]. NOD2 also responds to bacterial peptidoglycan and mediates the response to Gram-positive peptidoglycan, such as that from *Bacillus subtilis*. Again, digestion and fractionation of peptidoglycan identified specific fractions that simulated NOD2 [74].

NALP is a type of NOD-like receptor. NOD1 and NOD2 recognize intracellular bacterial cell products, but NALP3 responds to multiple stimuli to form a multiprotein complex termed the NALP3 inflammasome [2]. It is thought that NALP proteins sense inherent danger and link this with microbial products, creating a response mediated by the inflammasome that includes $K^+$ efflux and caspase-1 activation [75]. NALP3 is required for the secretion of IL-1$\beta$ and IL-18 that occurs when both bone marrow and peritoneal macrophages are stimulated with TLR7 ligands. NALP3 is required for caspase-1 activation, IL-1$\beta$ secretion, and cell death when macrophages are infected with the Gram-positive bacteria *Staphylococcus aureus* and *Listeria monocytogenes*, suggesting that it is involved in the response to specific bacterial pathogens, and may be limited to Gram-positive species [76]. Table 1 presents the overview of the NLRs and their PAMPs in human innate immunity.

*3.4. RIG-Like Receptors (RLRs).* Virus infection of mammalian cells triggers innate immune defenses through the PRRs for PAMPs within viral products that engage the intracellular signaling pathways to initiate an antiviral response. Viral RNA is a potent inducer of this host response and is recognized by specific TLRs or by cytoplasmic RNA helicases [62]. RLRs are intracellular receptors for RNA viruses. The RLR family is composed of at least 3 members: RIG-I, melanoma differentiation factor-5 (MDA5), and laboratory of genetics and physiology-2 (LGP-2). Studies of human cells defective in RIG-I signaling, or of cells from mice with a targeted deletion of RIG-I or MDA5, have revealed a remarkable degree of specificity of virus recognition between the individual helicases that could reflect differences in RNA binding and PRR function. Recognition by RLRs activates innate antiviral responses, mainly through the rapid induction of type I IFNs and inflammatory cytokines that limit viral replication and coordinate an antigen-specific, adaptive immune response [1, 2]. Each protein carries a helicase domain and a repression domain, and RIG-1 and MDA5 also possess two repeated N-terminal CARD domains. RIG-1 recognizes double-stranded RNA, activating IFN regulatory factor 3 (IRF3) and producing the key antiviral cytokines, type I IFNs [77].

MDA5 and RIG-I exhibit limited homology, with 23% and 35% homology in their CARD and helicase domains, respectively. LGP2 lacks a CARD domain, and the helicase domain has 31% and 41% homology with RIG-I and MDA5, respectively [78]. Activation of RIG-I or MDA5 increases IFN-$\beta$ secretion and activation of the IRF3 transcription factor, suggesting that these two RLR proteins activate the same signaling pathway [78]. LGP2 strongly inhibited the expression of an IFN-$\beta$ reporter gene and impaired IRF3 dimerisation, indicating that it has a negative regulatory role. Both MDA5 and LGP2 bind to double-stranded RNA. MDA5 and RIG-I, but not LGP2, reduced viral yields following infection with EMCV and VSV. RIG-I and MDA5, therefore, seem to be important in the IFN response to certain viruses, which seems to be, at least partly, a response to RNA [12]. Figure 1 presents the schematic overview of NOD1, NOD2 and NALP signaling pathways.

## 4. Pathogen Recognition Receptors in Dental Pulp and Periapical Tissues

The past decade has seen a rapid development of researches on innate immunity and PRR in dental pulp and periapical tissues (Table 2). The pulp has many MHC class II positive cells such as odontoblasts, pulp fibroblasts, and dendritic, endothelial, and neural cells, which are the most active antigen presenting cells (APCs) initiating immune responses to dental pathogens [34].

As we described previously, immune cells infiltrate into the odontoblastic layer close to a lesion where dentin is being destroyed by cariogenic bacteria. Thus, odontoblasts are the first cells encountered by pathogens entering dental pulp. Odontoblasts express TLR1–TLR6 and TLR9 but not TLR7, TLR8, and TLR10 [32, 53]. They recognize bacterial products including triacetylated lipopeptides (TLR2/TLR1) [42], diacetylated lipopeptides (TLR2/TLR6) [32], viral RNA (TLR3, TLR7, TLR8, and TLR9) [53], LPS (TLR4) [33, 50], flagellin (TLR5) [32], and unmethylated CpG DNA (TLR9) [32, 47]. TLR2 activation by LTA induces the differential production of certain proinflammatory cytokines, and it increases the ability of odontoblasts to recognize and respond to a wide variety of bacterial and viral by-products [32, 39, 42].

Once pulp inflammation initiated, various proinflammatory mediators and cytokines are upregulated in dental pulp, especially pulp fibroblasts. Recent researches demonstrated that TLR2, TLR3, TLR4, and TLR5 [31, 39, 40] and NOD1 and NOD2 [16, 38] are expressed by human pulp fibroblasts. TLR2 and TLR4 are expressed in various inflammatory cells and odontoblasts in inflamed pulp tissue [34, 36]. Activation of TLR2, TLR3, and TLR4 by their specific ligands induces the production of proinflammatory and chemokine proteins such as CCL2, CCL5, CCL7, CXCL8, and CXCL10 [40, 79]. TLR2 acts synergistically with NOD2 to stimulate proinflammatory mediator production in human pulp fibroblasts [39]. It also synergizes with the inflammation mediator (histamine receptor 1); thus fibroblasts express functional receptors that recognize pathogens and are potential initiators of immune/inflammatory events in dental pulp [40].

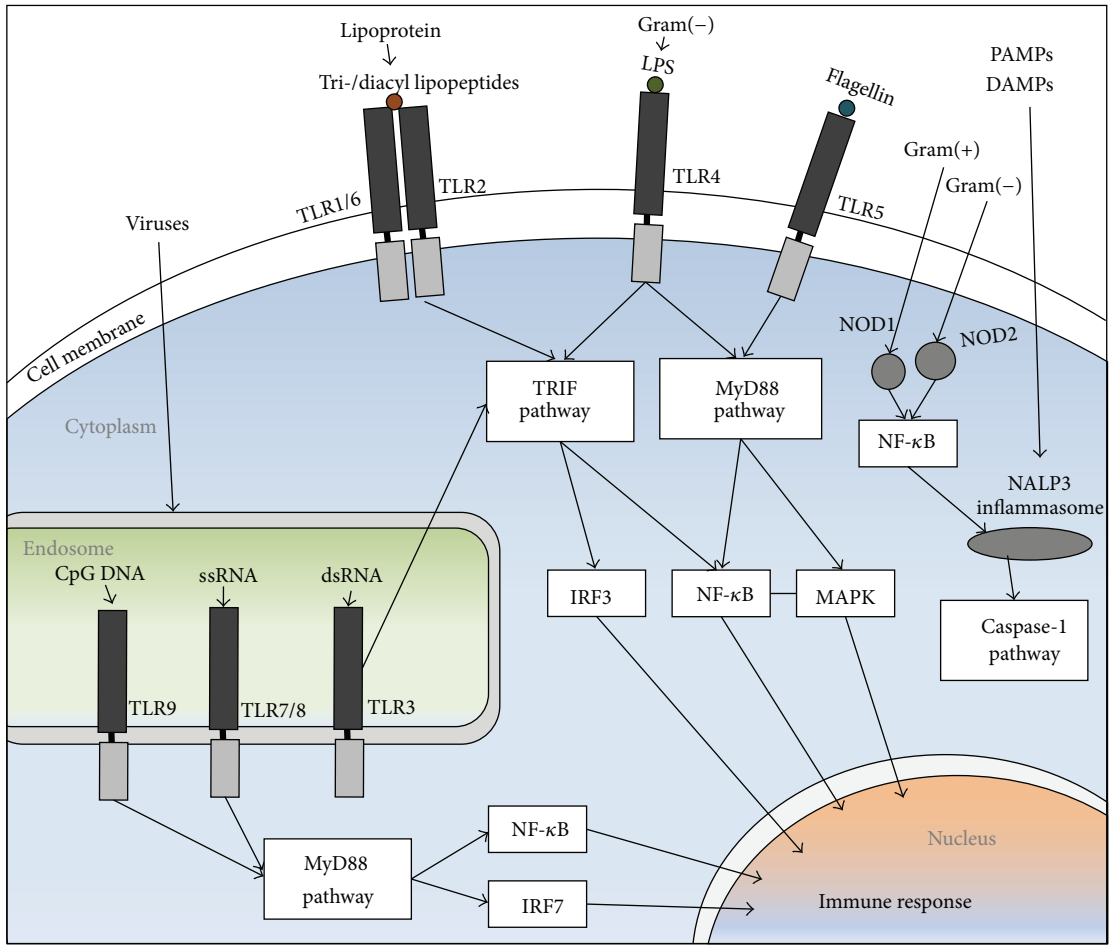


Figure 1: Schematic overview of TLR and NLR signaling pathways. PAMPs and DAMPs are recognized by PRRs. Heterodimer of TLR1/6+TLR2, TLR4, and endosomal TLR3 activate TRIF pathway, followed by induction of IRF and NF-$\kappa$B. TLR5 and endosomal TLR9 and TLR7 activate MyD88 pathway, followed by activation of MAPK, NF-$\kappa$B, and IRF7. NOD1 and NOD2 are cytoplasmic PRRs, and they trigger NF-$\kappa$B, and NALP3 inflammasome recruits and activates caspase-1 pathway. DAMP: damage-associated molecular patterns; IRF: IFN-regulatory factor; MAPK; mitogen-activated protein kinase; MyD88; myeloid differentiation primary-response gene 88; NF-$\kappa$B: nuclear factor-$\kappa$B; NALP3: NACHT, LRR, and pyrin domain containing protein 3; NOD: nucleotide-binding oligomerization domain; NLR: NOD-like receptor; PAMP: pathogen-associated molecular patterns; TLR: Toll-like receptor; TRIF: Toll/IL-1R (TIR) domain containing adaptor protein inducing IFN-$\beta$.

TLR2 is involved in detecting the Gram-positive bacterial components that dominate the microflora of failed root canal treatments. *Enterococcus faecalis* can survive in harsh environment and is known as one of the major etiologic factors in various stages of persistent periapical disease. *E. faecalis*, a Gram-positive facultative anaerobic bacterium, possesses antigenic LTA and lipopeptide components. These activate the TLR2/TLR1 complex in human odontoblasts. Transcription of inflammatory cytokines IL-8 and TNF-$\alpha$ is also increased [19, 49]. Chlorhexidine reduces the ability of LTA antigen to be recognized by TLR2, and this decreases the production of TNF-$\alpha$. Refractory periapical diseases contain large numbers of intraradicular Gram-positive bacteria [80].

TLR2 expression in various periapical lesions may play a role in the recognition of the atypical LPS of *P. gingivalis* [34]. T lymphocytes dominate the chronic periapical granuloma, and *in vitro* experiments showed that TLR2 is expressed in CD4+, CD3+, and CD14− T cells [81]. As mentioned above, TLR2 has an indirect role in adaptive immunity through the activation of APCs. However, its role extends to direct augmentation of antigen-specific Th1 responses. Sustained expression of TLR2 on memory T cells allows an immediate strong response on encountering a previously recognized pathogen [81]. Treg cells regulate and dampen Th cell-mediated immune reactions. Their activity may lead to various autoimmune diseases and inadequate development of an effective immune response during infection. Upon direct contact with bacterial ligands, TLR2 is expressed on Treg cells [82]. In periapical lesions, Treg cells produce TGF-$\beta$, which is responsible for inhibiting Th1-mediated cytokines. Exposure to TFG-$\beta$ abolishes the TLR2-mediated responses of odontoblasts [37].

Odontoblasts and pulp fibroblasts express TLR4 in response to antigen challenge. In inflamed pulp model, TLR4 expression on pulp macrophage and dendritic-like cells was lower and slower compared to that of TLR2. Root canal

Table 2: Summary of recent finding with PRR in dental pulp.

| Author/year/journal | PRR | Cell/animal | Study design | Relevant findings |
|---|---|---|---|---|
| Durand et al., 2006, J Immunol [32] | TLR | Odontoblast | *In vitro* | LTA upregulates TLR2 and chemokine expression while downregulating dentin matrix synthesis and mineralization |
| Jiang et al. 2006 J Endod [33] | TLR4 | Odontoblast Dental pulp tissue | *In vitro* | TLR4 expression in normal DP |
| Mutoh et al., 2007, J Endod [34] | TLR2, TLR4 | Murine pulp tissue | *In vivo* | TLR2, TLR4 expression in DP TLR2 regulates early stage of pulp inflammation |
| Marcato et al., 2008, Oral Microbiol Immunol [35] | TLR2, TLR4 | Mouse model | *In vivo* | TLR2, TLR4 induce NO and ROS production by macrophage stimulated with root canal pathogens |
| Mutoh et al., 2009, J Endod [36] | TLR2, TLR4 | SCID mice | *In vivo* | TLR2, TLR4 are triggered by dental pathogen in irreversible pulpitis |
| Horst et al., 2009, J Dent Res [37] | TLR2, TLR4 | Odontoblast | *In vitro* | TGF-$\beta$1 inhibits TLR2, TLR4 expression against dental pathogens |
| Hirao et al., 2009, J Dent Res [16] | TLR2, TLR4 and NOD1, NOD2 | HDPF | *In vitro* | TLR2, TLR4, NOD1, and NOD2 expression in DP NOD2 is an immunomodulator through TLR2, leading to progressive pulpitis |
| Lin et al., 2009, J Endod [38] | NOD2 | HDPC | *In vitro* | NOD2 expression in normal DP |
| Keller et al., 2010, Immunobiology [39] | TLR2 | Odontoblast HDPF | *In vitro* | LTA upregulates TLR2 in odontoblasts and HDPF |
| Park et al., 2010, J Dent Res [40] | TLR2 | HDPF | *In vitro* | TLR2 on HDPF with histamine receptor-1 induces pulpal inflammation via Cox-2 activation |
| Botero et al., 2010, J Dent Res [41] | TLR4 | HDPSC (HDPF) | *In vitro* | LPS upregulates VEGF DPSC express TLR4 |
| Farges et al., 2011, Immunobiology [42] | TLR2 | Odontoblast | *In vitro* | TLR2 engages production of mediators in odontoblasts |
| Keller et al., 2010, Innate Immun [43] | TLR2, NOD2 | HDPC, odontoblast | *In vitro* | Upregulation of TLR2, NOD2 through stimulation via LTA in inflamed DP |
| Lee et al., 2011, J Endod [18] | NOD1 | HDPF | *In vitro* | Upregulation of NOD1 in inflamed DP |
| Song et al., 2012, J Endod [44] | NALP3 | HDPF | *In vitro* | NALP3 upregulates in dental pulp immune defense |
| Da Silva et al., 2012, J Endod [45] | TLR2 | TLR2 KO mice | *In vivo* | TLR2 regulates inflammatory response and host's immune to root canal and periradicular infection |
| Carrouel et al., 2013, J Endod [46] | TLR2 | Odontoblast | *In vitro* | LBP reduces TLR2-dependant immune responses by LTA in human odontoblast-like cells |
| Zhang et al., 2013, Int Endod J [47] | TLR9 | Odontoblast | *In vitro* | TLR9 regulates the remodeling of injured DP and hard tissues by inducing MMP-13 |
| He et al., 2013, Int Endod J [5] | TLR4 | HDPSC | *In vitro* | LPS upregulates IL-8 with engagement of TLR4/MyD88/NF-$\kappa$B and MAPK pathways in DP |
| Keller et al., 2011, Innate Immun [43] | NOD2 | Odontoblast | *In vitro* | LTA augmented NOD2 expression in odontoblasts |
| Wang et al., 2013, J Endod [48] | AIM2 | Rat model Rat pulp cell | *In vivo* *In vitro* | AIM2 is only detected in the odontoblast layer and mediates inflammatory response during pulpitis |
| Cardoso et al., 2014 J Endod [49] | TLR2 | Inflamed and healthy human dental pulp tissue | *In vitro* | Hypomethylation of TLR2 and CD14 gene mediates immune responses against LPS |

Table 2: Continued.

| Author/year/journal | PRR | Cell/animal | Study design | Relevant findings |
|---|---|---|---|---|
| He et al., 2014 J Endod [50] | TLR4 | HDPSC | *In vitro* | LPS enhances Wnt5a expression via TLR4/MyD88/NF-$\kappa$B pathways in DP |
| Feng et al., 2014, Cell Tissue Res [51] | TLR4 | HDPSC | *In vitro* | LPS+ TLR4 complex stimulates inflammation in DP |
| Liu et al., 2014, J Endod [52] | TLR4 | HDPSC | *In vitro* | LPS activates TLR4<br>TLR4 regulates the proliferation and migration of DPSC in deep dental caries |
| Pääkkönen et al., 2014, Int Endod J [53] | TLR3, TLR7, TLR8, and TLR9 | Odontoblast | *In vitro* | TLR3, TLR7, TLR8, and TLR9 mRNA (virus recognition PRR) participate in immune response in DP |
| Lee et al., 2014, Clin Oral Invest [54] | TLR2, TLR4, and NALP3 | HDPC | *In vitro* | TLR and NALP3 activate immune responses during progression of pulpitis |
| Zhang et al., 2015, Mol Immunol [55] | NALP3, TLR4 | HDPF | *In vitro* | NALP3 in HDPFs triggers IL-1 secretion in response to LPS plus ATP<br>LPS engaged TLR4/MyD88/NF-$\kappa$B pathway to enhance NLRP3 |
| Lee et al., 2014, Clin Oral Invest [54] | NALP3 | HDPF | *In vitro* | NOD2 activates TLR2, TLR4, and NALP3 inflammasome-signaling pathways |
| Liu et al., 2014, Int Endod J [52] | NALP3 | HDPSC | *In vitro* | NALP3 expressed in periapical lesion |

TLR: Toll-like receptor; LTA: lipoteichoic acid; DP: dental pulp; NO: nitric oxide; ROS: reactive oxygen species; SCID: severe combined immunodeficiency mice; TGF-$\beta$1: transforming growth factor-$\beta$1; HDPF: human dental pulp fibroblast; HDP(S)C: human dental pulp (stem) cell; NOD: nucleotide-binding oligomerization domain; Cox: cyclooxygenase; LPS: lipopolysaccharide; VEGF: vascular endothelial growth factor; DPSC: dental pulp stem cell; NALP: NACHT [neuronal apoptosis inhibitory protein (NAIP), CIITA, HET-E, and TP-1]; KO: knockout; LBP: lipopolysaccharide-binding protein; MMP: matrix metalloproteinase; MyD88: myeloid differentiation factor 88; NF-$\kappa$B: nuclear factor kappa B; MAPK: mitogen-activated protein kinase; AIM: absent in melanoma; ATP: adenosine triphosphate.

pathogens stimulate TLR2 and TLR4, and they participate in the induction and progression of periapical lesion through NO and ROS production by activated macrophages [35]. TLR4 is involved in detecting the Gram-negative bacterial component LPS (lipid-A portion). *P. gingivalis* is often retrieved from infected root canal systems. The lipid-A subunit of LPS obtained from *P. gingivalis* has several different structures. Although TLR2 does not play an active role in the recognition of Gram-negative bacteria, heterogeneous LPS can activate host immune cells through a TLR2-dependent pathway [34, 83]. Botero et al. reported that LPS is associated with recognition of TLR2 and TLR4, and it induces vascular endothelial growth factor (VEGF) expression in dental pulp via MAPK activation [41]. TLR4 was detected in the early stage of pulp inflammation in experimentally inflamed pulps in mouse model [36].

NLRs share common features with TLRs in that ligand binding is mediated by LRR domain. Hirao et al. demonstrated that human pulp fibroblasts constitutively express intracellular NOD1 and NOD2 as well as TLR2 and TLR4, and each PRR-specific ligand was upregulated to produce various proinflammatory mediators, suggesting that NODs have a potent influence on proinflammatory responses in dental pulp [16]. NOD1 and NOD2 participate in the innate immune response through the NF-$\kappa$B pathway, and NOD1/NOD2 signaling has been reported to trigger IL-8 expression. NOD1 and NOD2 are expressed in normal dental pulp, and their expression is upregulated in inflammatory responses [6, 18, 38]. NOD2 participated in the odontoblast differentiation via downregulation of MAPKs and osteoclastogenesis by providing macrophage colony-stimulating factor (M-CSF) and receptor activator of NF-$\kappa$B ligand (RANKL) in the presence of muramyl dipeptide (MDP) [6, 43]. MDP also activates NOD2-specific si-RNA, followed by upregulation of the TLR2, TLR4, and NALP3 signaling pathways in dental pulp cells to trigger the various inflammatory mediators and cytokines, which enhance pulp immune responses against dental pathogens [54].

NALP3 is expressed in human dental pulp cells and in the inflammatory cells and pulp fibroblasts of inflamed pulp, which points to an important role for NALP3 in the recognition of invading pathogens and the initiation of immune responses [44]. The NALP3 inflammasome in pulp fibroblasts is crucial for IL-1$\beta$ secretion in response to LPS, and the latter triggers the TLR4/NF-$\kappa$B pathway to enhance NALP3 levels in a ROS-dependent manner [55].

Invasion of bacteria or their by-products into the periapical region from an infected root canal system leads to inflammatory reactions that involve various host-derived cells, antibodies, complement and cytokines, and an array of inflammatory mediators, which may cause local tissue destruction in the bone around the periapical tissues, and root resorption. PRR expression is not restricted to macrophages and DCs, in which they have been mainly studied, but includes a variety of cell types, including the gingival fibroblasts that make up the majority of cells in periodontal tissues [84]. Recent research has demonstrated upregulation of TLR1, TLR2, TLR4, and TLR5 at the cell surface and of TLR3, TLR7, TLR8,

and TLR9 and NOD1 and NOD2 intracellularly in human gingival fibroblasts [85]. Stimulation of gingival fibroblasts with TLRs and NODs induced the inflammatory cytokines IL-6 and IL-8, an indication that these receptors are active in periodontal tissue. Cementoblasts express TLR4 in response to LPS resulting in alteration of gene expression related to cementum formation, upregulation of osteoclastogenesis-associated molecules such as receptor activator of NF-$\kappa$B ligand (RANKL) [86]. Stimulation of gingival fibroblasts with TLRs and NODs induced the inflammatory cytokines IL-6 and IL-8, an indication that these receptors are indeed active in periodontal tissue [85]. It is also proved that NALP3 is expressed in the inflammatory periapical tissues [52].

Inflammatory periapical lesions are initiated by polymicrobial infections by Gram-positive and Gram-negative bacteria; they are maintained and exacerbated by prolonged bacterial activity and by their by-products derived from infected root canal systems. Of the various innate and adaptive immune cells found in periapical lesions, most have migrated to the site from the peripheral blood in response to antigens, rather than residing in healthy periapical tissues [45, 81].

## 5. Conclusion

The entry of dental pathogens into dental pulp evokes multiple modes of PRR activation in response to PAMPs. TLRs play a key role in the innate immune system, and ten TLR family members are present in humans (TLR1–TLR10). These differ in their sites of expression and/or ability to recognize different PAMPs. TLRs trigger activation of signaling pathways involving MyD88 and TRIF that lead to the production of proinflammatory cytokines and chemokines via the NF-$\kappa$B pathway. NLR families include NOD1, NOD2, and NALP3. NLRs are intracellular receptors that recognize PAMPs that have entered the cell and also danger-associated molecular patterns (DAMPs), which are induced during cellular stress. Different levels of NOD1 and NOD2 are activated depending on which pathogenic species is recognized. The ability of PRRs to recognize diverse groups of PAMPs allows the host immune system to respond to encounters with a variety of dental pathogens. Future research needs to clarify the signal transduction pathways subsequent to activation of the PRRs and methods for interfering with PRR activation and their potential therapeutic applications.

## Conflict of Interests

The authors deny any conflict of interests regarding the publication of this paper.

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*Clinical Study*

# Sensory Neuropeptides and Endogenous Opioids Expression in Human Dental Pulp with Asymptomatic Inflammation: *In Vivo* Study

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*Purpose*. This study quantified the expression of substance P (SP), calcitonin gene-related peptide (CGRP), $\beta$-endorphins ($\beta$-End), and methionine-enkephalin (Met-Enk) in human dental pulp following orthodontic intrusion. *Methods*. Eight patients were selected according to preestablished inclusion criteria. From each patient, two premolars (indicated for extraction due to orthodontic reasons) were randomly assigned to two different groups: the asymptomatic inflammation group (EXPg), which would undergo controlled intrusive force for seven days, and the control group (CTRg), which was used to determine the basal levels of each substance. Once extracted, dental pulp tissue was prepared to determine the expression levels of both neuropeptides and endogenous opioids by radioimmunoassay (RIA). *Results*. All samples from the CTRg exhibited basal levels of both neuropeptides and endogenous opioids. By day seven, all patients were asymptomatic, even when all orthodontic-intrusive devices were still active. In the EXPg, the SP and CGRP exhibited statistically significant different levels. Although none of the endogenous opioids showed statistically significant differences, they all expressed increasing trends in the EXPg. *Conclusions*. SP and CGRP were identified in dental pulp after seven days of controlled orthodontic intrusion movement, even in the absence of pain.

## 1. Introduction

Asymptomatic inflammation (AI) (also known as silent, low-grade, or painless inflammation) is a concept that describes a scenario in which an etiologic agent is present without clinical evidence of harm or pain. It can be found in different types of cancer [1], genital tract diseases [2], cerebral infarction [3], or diabetic conditions [4]. The oral environment is also vulnerable to suffer AI under certain pathologies, including chronic apical periodontitis [5] and periodontal disease [4]. Dental treatments such as deep restorations or orthodontic movements [6] can cause incessant injury to dental tissues that are not identified as noxious. Irreversible pulpitis can also occur asymptomatically [7, 8], which may progress to dental pulp necrosis without treatment [9].

Vascular, neural, cellular, and biochemical changes from inflammation can be present in the absence of pain. Neuropeptides released from primary afferent neurons (PAN) are crucial factors in provoking inflammation of neural origin or "neurogenic inflammation" [10]. Substance P (SP) and calcitonin gene-related peptide (CGRP) are capable of inducing vasodilation, plasma extravasation, immune cell chemotaxis, and pain [11, 12]. Controversy exists when both neuropeptides are expressed asymptomatically. This evidence is particularly low in dental pulp studies.

Pain can be modulated by central and peripheral nervous mechanisms. Peripherally, opioid-containing immune cells (OCIC) play the main role due to $\beta$-endorphins ($\beta$-End) and methionine-enkephalin (Met-Enk) release [13]. These substances induce the activation of opioid receptors (OR) located in PAN [14], thus causing electrical changes that modulate pain partially via the inhibition of neuropeptide release [15, 16].

Considering both sides, it is interesting to analyze these two families of peptides within dental pulp, using controlled orthodontic forces that may allow nerve response without causing clinical symptomatology [17]. The aim of this study was to determine the expression levels of SP, CGRP, $\beta$-End, and Met-Enk in human dental pulp following orthodontic intrusion.

## 2. Material and Methods

*2.1. Patients.* Eight patients of both sexes who were between 12 and 16 years old were selected. Patient recruitment was performed at the Endodontics and Pediatric Dentistry Clinics. Inclusion criteria were as follows: being systemically healthy and indicated for the extraction of the four first premolars for orthodontic reasons; teeth without caries, fractures, or periodontal disease; and teeth with radiographically evident complete radicular formation. Exclusion criteria were as follows: patients receiving recent anti-inflammatory, analgesic, or antibiotic treatment; those who had root resorption (from any cause), permanent or provisional restorations in the first premolars or radicular dilacerations; smokers; pregnant patients; and those who have occlusal disorders.

*2.2. Study Design.* A descriptive comparative pilot study was conducted with the approval of the Institutional Ethics Committee (Approval Code number CEIFE-002-010), according to the Declaration of Helsinki. Written informed consent was explained and obtained from parents/legal guardians of each patient. Intrusive orthodontic appliances were designed using standard $0.018''$ orthodontic brackets and double tubes (Sybron/Ormco, Orange, CA, USA), selected for the first premolars and permanent first molars, respectively. Both brackets were bonded to each tooth using the adhesive system Prime & Bond (3M Unitek, Monrovia, CA, USA) and composite Filtek 350 (3M Unitek), standardizing their positions with an Anderson calibrator (Dentaurum GmbH, Ispringen, Germany). Using number 139 orthodontic pliers (Dentaurum GmbH), a 1 mm loop was formed with a retentive fold on the opposite side of a stainless steel wire ($0.018'' \times 0.025''$). The customized wire was placed in the tube slot and adjusted to allow the loop to be bent until an acute angle was achieved. Using a dynamometer (Corex, Haag-Streit, Kowniz, Switzerland), the angulation was standardized at 40° to allow for a 150–200 g intrusive force.

Two premolars from each patient were randomly selected and assigned to two different groups: the asymptomatic inflammation group referred to as experimental group, which would undergo controlled intrusive force for seven days (EXPg), and the control group (CTRg), used to determine the basal levels of each substance. Each patient was scheduled to obtain the samples from the control tooth before any clinical intervention took place. Each control tooth was anesthetized with 4% prilocaine (Pricanest, Ropsohn Therapeutics, Bogotá, Colombia). From that moment, the entire sampling procedure was performed within a 10-minute period. After extraction, a second assistant created a 1 mm longitudinal groove over the vestibular tooth surface using a high-speed diamond bur with copious irrigation to facilitate the mechanical fracture of the tooth and the intact acquisition of pulpal tissue, which was immediately stored in a tagged cryovial containing 1.8 mL of 4% paraformaldehyde. The cryovial was stored at −70°C. The orthodontic device assigned to the EXPg was then placed. After its activation, each patient received instructions to call if any problem or severe discomfort was experienced and, if necessary, to take 400 mg of ibuprofen as a rescue analgesic.

For the following six days, the patients were contacted by telephone to evaluate their progress along the experimental period. Information on their symptoms, analgesic usage, or orthodontic device displacement was recorded. At day seven, patients were scheduled to obtain the dental pulp sample from EXPg, as described above.

*2.3. Radioimmunoassay.* A radioimmunoassay (RIA) was performed to quantify the amount of each substance obtained from each sample, according to previous investigations reported [18]. SP, CGRP, $\beta$-End, and Met-Enk release were determined by competition RIA binding assays using a human SP RIA-kit (reference RK-061-05), a human CGRP RIA-kit (reference RK-015-02), a human $\beta$-End RIA-kit (reference RK-022-14) (Phoenix Peptide Pharmaceuticals, Burlingame, CA, USA), and a human Met-Enk RIA-kit (reference S-2119) (Peninsula Laboratories LLC, Bachem Group, San Carlos, CA, USA). For each kit, 100 $\mu$L of antiserum and 100 $\mu$L of various neuropeptide/opioid concentrations (1–128 pg $\mu L^{-1}$) or 100 $\mu$L of dental pulp tissue extracts were incubated in polypropylene tubes at room temperature for 20 hours. Then, 100 $\mu$L of radioactive 125I tracer was added and left to incubate for another 24 hours. Bound fractions were precipitated by the addition of 100 $\mu$L of a secondary antibody (goat anti-rabbit immunoglobulin G serum), 100 $\mu$L normal rabbit serum, and 500 $\mu$L RIA buffer containing 1% polyethylene glycol 4,000. After 2 hours of incubation at room temperature, the suspensions were spun at 3,500 rpm (4,000 g) for 40 minutes at 4°C to precipitate the bound fractions. The supernatants were carefully aspirated, and pellet radioactivity was read on a gamma counter (Model B5002, Packard Instrument International, Zurich, Switzerland). All samples were assayed in duplicate, and the mean values were calculated. Finally, Scatchard analysis of the binding data was used to assess the amount of neuropeptide/opioid present in each sample.

*2.4. Statistical Analyses.* The results were analyzed by the Mann-Whitney $U$ test to compare the differences among groups for continuous variables. A difference was considered to be significant if the probability of its occurring by chance alone was <5% ($P < 0.05$) in a two-tailed test.

Table 1: Expression levels for substance P (SP), calcitonin-gene related peptide (CGRP), $\beta$-endorphins ($\beta$-End), and methionine-enkephalin (Met-Enk) in the control group (CTRg) and the asymptomatic inflammation group (EXPg).

| Group | Neuropeptides/endogenous opioids | | | |
|---|---|---|---|---|
| | SP | CGRP | $\beta$-End | Met-Enk |
| CTRg | 83.51 ± 11.35 | 13.73 ± 2.84 | 12.44 ± 1.74 | 2.29 ± 0.84 |
| EXPg | 145.93 ± 119.26 | 22.46 ± 3.06 | 16.51 ± 8.95 | 10.74 ± 13.78 |
| $P$ value | <0.05* | <0.05* | >0.05 | >0.05 |

Data are presented in picograms of each substance per milligram of dental pulp simple.
*Statistically significant difference.

## 3. Results

The results are shown in Table 1. In CTRg basal levels of both neuropeptides and endogenous opioids were observed. In the EXPg, SP and CGRP exhibited statistically significant different levels ($P < 0.05$). Although none of the endogenous opioids showed statistically significant differences ($P > 0.05$), they all expressed increasing trends in the EXPg. None of the patients enrolled were eliminated from the study because no one reported severe pain episodes or the need to take the rescue analgesic treatment. All patients reported tolerable discomfort localized in the tooth assigned to EXPg, for the two or three days after orthodontic activation. By day seven, all of the symptoms were absent, and all of the orthodontic devices were still active.

## 4. Discussion

Mechanisms related to neurogenic inflammation and pain modulation in AI are not fully understood. In this study, the expression of two somatosensory neuropeptides and two OPs in dental pulp AI caused by orthodontic intrusion was determined. This clinical model allowed us to obtain a biphasic inflammatory transition, which began with discomfort during the acute phase and finally achieved an asymptomatic state [6]. The intrusion rate of the teeth was not evaluated clinically. Since the intrusion period of the experiment was seven days, it was considered not objective to make a clinical measurement of the distance. In this experiment, intrusion stimulus was only measured in function of time. Although the majority of available evidence is focused on the biochemistry of painful conditions, the study of AI is a promising field that may help explain how important diseases appear after the development of "anonymous" inflammation. In this study, the presence of an initial inflammatory tenderness was required to assess the beginning of the asymptomatic phase; it was a requirement for this study that all patients report no presence of pain when the samples were obtained.

Orthodontic movements are able to induce the local release of SP and CGRP. Previous studies have demonstrated, within dental pulp, the expression of markers of neurogenic inflammation (substance P and CGRP) using the model of inflammation employed in this study; this controlled orthodontic movement was selected as a well-standardized available model for human clinical trials focused on inflammation [6, 19–21]. Chemotaxis of the macrophage population induced by neuropeptides in late orthodontic inflammation [22] is crucial to establish the neuropeptidergic and OP relationship. Furthermore, nerve fiber sprouting and neurogenic inflammation are common during pulpal insult, including early force application during orthodontic tooth movement [23]. Macrophages express NK1 and CGRP1/2 receptors, which are activated by SP and CGRP, respectively [11], favoring their activation [15, 24]. These cells are the main secretors of OP [25], including $\beta$-End and Met-Enk [26]. Interestingly, under inflammatory conditions, PAN, the main source of local SP and CGRP release [14], exhibits an upregulation expression of OR [13, 27], which can be targeted by macrophage analgesic products (Figure 1). Controversy surrounds the fact that macrophages are also capable of secreting somatostatin (a substance able to cause analgesia); thus, pain control may not be exclusively caused by OP. However, the local presence of SP selectively inhibits somatostatin release but not $\beta$-End [24].

These results demonstrate an important increase of SP and CGRP levels in EXPg ($P < 0.05$). The release of sensory neuropeptides occurs almost immediately, in a manner described as an "axonal reflex" [22]. Such a response supports the role of neurogenic inflammation of the dental pulp after low-grade orthodontic continuous movement [20, 22, 28]. Both neuropeptides are frequently associated with pain from pulpal origin [29–31]; however, they have also been identified in asymptomatic irreversible pulpitis [32]. The importance of the role of neuropeptides in inflammation was previously studied by determining their absence, not their presence. Different denervation experiments showed how the deprivation of the sensory nerve supply can attenuate the local inflammatory response [9].

$\beta$-End and Met-Enk showed an increase for EXPg. Both OPs are postulated to play the leading role in endogenous antinociception [33] due to their action as negative regulators of neurogenic inflammation [16] by the inhibition of SP and CGRP release [14, 15]. However, this study shows that, after orthodontic intrusion, low levels of OP were not enough to attenuate neuropeptide expression.

It is important to consider why late neurogenic inflammation can develop in the absence of pain. Current evidence offers possible explanations. First, dental pulp is able to adjust slowly to low-grade inflammation, assimilating higher levels of proalgesic mediators in the absence of pain [9]. Second, experimental tooth movement is capable of activating central trigeminal pain modulation mechanisms [34], causing a reduction in the local recruitment of OCIC to peripheral injured tissues; thus, the net effect will be the control of pain and lower peripheral opioid peptide concentration [15, 25, 35–37]. Third, new evidence explores the dual effects of neuropeptides in relation to pain experience. SP enhances opioid release, and it is possible that its N-terminal fragment may act as a miu-opioid receptor (MOR) agonist once its expression is upregulated during inflammation. Both observations may explain the lack of effectiveness of NK1 antagonists to modulate pain [38]. For CGRP, the antinociceptive effect is related to the upregulation of MOR when it is applied

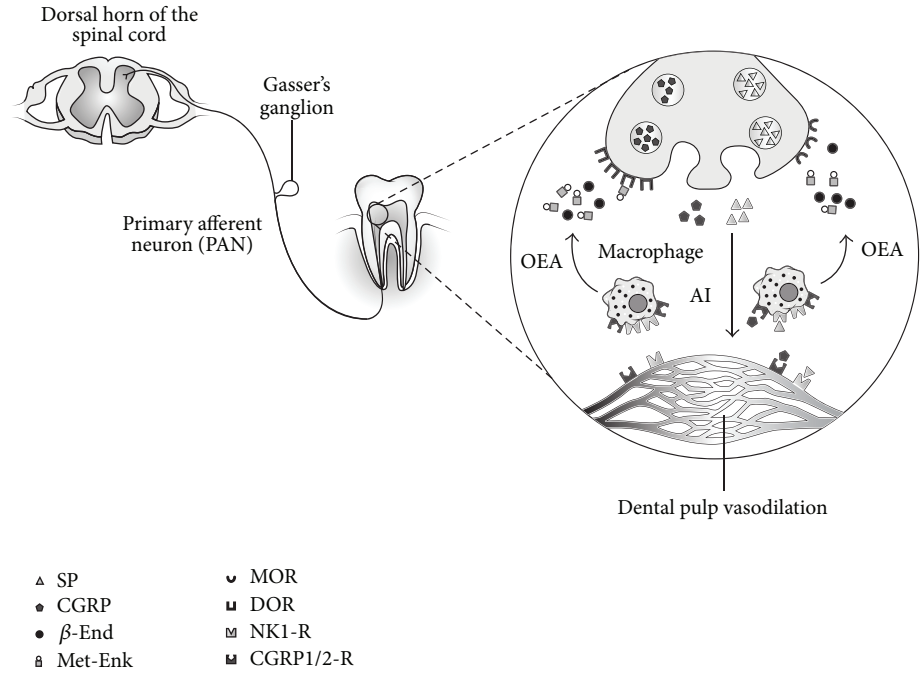


Figure 1: Proposed relationship between neurogenic inflammatory and endogenous opioid analgesic mechanisms. SP: substance P, CGRP: calcitonin gene-related peptide, $\beta$-End: $\beta$-endorphin, Met-Enk: methionine-enkephalin, NK1-R: neurokinin 1 receptor, CGRP1/2-R: calcitonin gene-related peptide receptors 1 and 2, MOR: $\mu$-opioid receptor, DOR: $\delta$-opioid receptor, OEA: opioid endogenous analgesia, AI: asymptomatic inflammation, and PAN: primary afferent neuron.

centrally [38] and peripherally by the local suppression of interleukin-2 production, causing an anti-inflammatory effect [28]. Finally, though both neuropeptides are strong vasodilators, this effect may be masked by low blood flow after orthodontic intrusion, thus avoiding the increase of inner pressure in a low compliance pulpal environment [39–41]. All of these hypotheses must be evaluated in further studies, including more patients in order to confirm these findings, as well as new complementary experiments including pulp tissue immunostaining to evaluate neurons, peptides activity, and immune and inflammatory cells participation. Also, it is necessary to include peptides measurement at different time points in order to elucidate the possible mechanism of asymptomatic inflammation.

## 5. Conclusions

In this preliminary report, SP and CGRP were identified in dental pulp after seven days of controlled orthodontic intrusion movement, even in the absence of pain. At the same time, endogenous opioids exhibited no statistical differences compared with control levels; however, an increase tendency was appreciable for both.

## Ethical Approval

This study was registered and approved by the Institutional Ethics Committee of the Faculty of Dentistry at San Luis Potosi University, San Luis Potosi, Mexico (Approval Code number CEIFE-002-010).

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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*Review Article*

# Is Pulp Inflammation a Prerequisite for Pulp Healing and Regeneration?

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The importance of inflammation has been underestimated in pulpal healing, and in the past, it has been considered only as an undesirable effect. Associated with moderate inflammation, necrosis includes pyroptosis, apoptosis, and nemosis. There are now evidences that inflammation is a prerequisite for pulp healing, with series of events ahead of regeneration. Immunocompetent cells are recruited in the apical part. They slide along the root and migrate toward the crown. Due to the high alkalinity of the capping agent, pulp cells display mild inflammation, proliferate, and increase in number and size and initiate mineralization. Pulp fibroblasts become odontoblast-like cells producing type I collagen, alkaline phosphatase, and SPARC/osteonectin. Molecules of the SIBLING family, matrix metalloproteinases, and vascular and nerve mediators are also implicated in the formation of a reparative dentinal bridge, osteo/orthodentin closing the pulp exposure. Beneath a calciotraumatic line, a thin layer identified as reactionary dentin underlines the periphery of the pulp chamber. Inflammatory and/or noninflammatory processes contribute to produce a reparative dentinal bridge closing the pulp exposure, with minute canaliculi and large tunnel defects. Depending on the form and severity of the inflammatory and noninflammatory processes, and according to the capping agent, pulp reactions are induced specifically.

## 1. Introduction

The alternative stages of dental pulp inflammation were restricted for many years with two limited options: necrosis or apoptosis. They appeared to be closely associated with four cardinal signs, reported in many references found in the literature as rubber, dolor, color, and tumor (swelling). Several small molecules and proteins are normally kept within the cells. In these areas, extensive cell death and tissue necrosis, also called coagulation necrosis, may also occur. More recently, a cascade of four stages was identified. There is actually a need for redefinitions of the physiopathological events, which might occur. The dental pulp may be exposed to the carious lesion or influenced by the adverse effects of filling materials (Figure 1). The inflammatory processes are gradually increasing from mild (moderate) to severe inflammation. Subjected to necrosis or apoptosis, nemosis has been recently added to the list of processes implicated in the destruction of the dental pulp [1, 2]. Pulp healing is the first step, followed by regeneration. This cascade of events is directly linked to the deleterious effects of inflammation processes in the presence or absence of pulp remnants.

The repair of dental pulp by direct capping with calcium hydroxide [$Ca(OH)_2$] or by implantation of bioactive extracellular matrix (ECM) molecules implies four sequential steps: a moderate inflammation, the commitment of adult reserve stem cells, their proliferation, and terminal differentiation [3] (Figure 2). Most of the published studies report that the healing sequence starts with an initial moderate inflammatory process, and now there are evidences that inflammation is a prerequisite for tissue healing as a first step, followed by pulp regeneration, also described as pulp repair.

## 2. Mechanisms Implicated in Pulp Inflammation

*2.1. Inflammation.* The importance of inflammation in pulp healing has been underestimated, for a long time considered only as an undesirable effect, leading in most cases to pulp

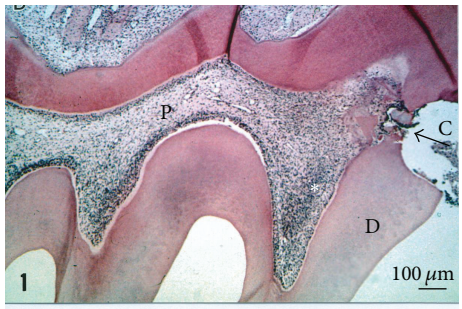



Figure 1: Pulp exposure and moderate inflammatory process. A cavity (C) was drilled on the mesial aspect of the six-week-old rat's first maxillary molar. One week after the pulp exposure, dentin debris is pushed in the pulp exposure. A moderate inflammatory reaction is seen in the mesial pulp horn (white asterisk). Hematoxylin-eosin staining. P = pulp. Bar = 100 $\mu$m.

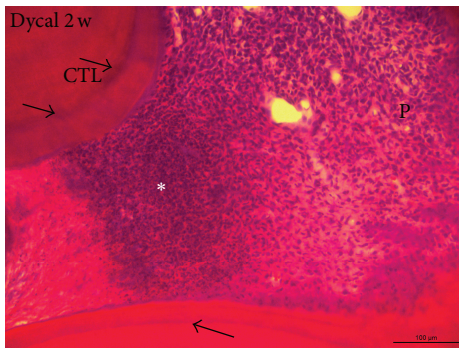



Figure 2: Pulp capping with calcium hydroxide (Dycal). Two weeks after the direct capping of a calcium hydroxide (Dycal), within the pulp calciotraumatic lines (CTL) (arrows), separate the dentin formed before the preparation of the cavity from the reactionary dentin (RD). In the left part, the $Ca(OH)_2$ has induced the formation of a reparative bridge in the isthmus separating the central from the distal pulp horns of 6-week-old rat's maxillary molar. On the left part of the pulp, the necrotic tissue is acellular, whereas in the right part of pulp, the vital pulp displays proliferating inflammatory cells (white asterisk). In the right part of the figure, pulp cells (P) differentiate and form odontoblast-like cells. Alizarin red staining. Bar = 100 $\mu$m.

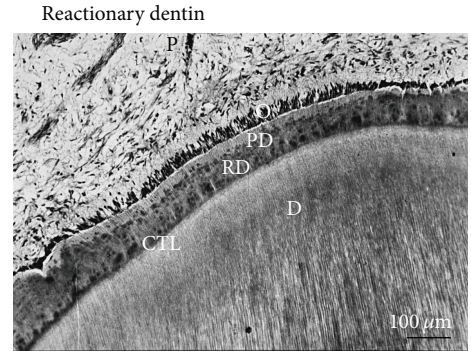



Figure 3: Reactionary dentin formation beneath a calciotraumatic line. A calciotraumatic line (CTL) separates the tubular secondary dentin (D) from the reactionary dentin (RD) formed in response to the treatment of carious lesion of human premolar (young adult). Odontoblasts (O) located in the outer layer of the pulp (P) synthetize and secrete the components of predentin (PD). Hematoxylin-eosin staining. P: pulp. Bar = 100 $\mu$m.

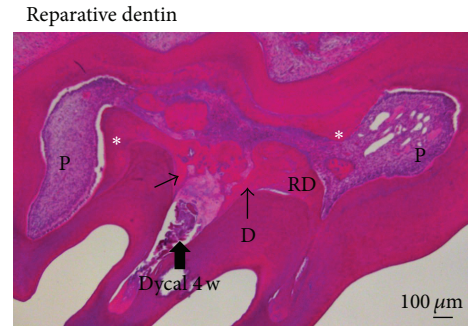



Figure 4: Formation of a reparative dentinal bridge. Six-week-old rat's first maxillary molar, followed by pulp capping with Dycal, 4 weeks after $Ca(OH)_2$ implantation within the pulp exposure (thick arrow). The dentinal reparative bridge (arrows) is still incomplete. Tunnels and other defects connect the oral cavity and the dental pulp. Along the walls of the pulp chamber, a dense and continuous layer of reactionary dentin (RD and white asterisks) is formed, reducing the pulp (P) volume. Hematoxylin-eosin staining. Bar = 100 $\mu$m.

necrosis and other adverse consequences. In view of a series of recent results, the inflammatory process should be reexamined to understand the potential and the beneficial effects of this process [3]. Altogether, these studies pave the way for a better understanding of the initial molecular and cellular events leading to pulp repair, as well as the development of the ideal materials to promote pulpal healing [3]. Partial pulpotomy after limited pulp capping, or total pulpotomy (namely, in deciduous teeth), and direct or indirect pulpectomy in permanently immature or older teeth constitute a whole range of clinical options [4]. The effects of $Ca(OH)_2$ containing pulp capping agents on pulp cell migration, proliferation, and differentiation have been specified [5]. $Ca(OH)_2$ induces beneficial effects due to chemical injury caused by the hydroxyl ions. A limited necrosis is induced against the vital pulp tissue (Figure 2). Necrosis provokes a slight irritation and stimulates pulp repair. Vascular and inflammatory cell migration and proliferation control mesenchymal and endothelial pulp cells, and also the formation of collagen (Figure 3). Odontoblasts differentiate and contribute to the formation and mineralization of a reparative dentinal bridge. Dentinal bridge develops following direct pulp capping. Tunnel defects favor the diffusion of bacteria issued from the oral cavity, which penetrate into the pulp (Figure 4). They contribute to microbial recontamination due to the numerous osteoblasts present in the reparative osteodentin bridge [6]. Inflammation of the tooth has been considered mostly as a negative factor leading to pulp destruction by necrosis or apoptosis. In short-term experiments, 1, 3, or 7 days after amelogenin implantation (A + 4 or A − 4), Osteopontin (OPN), which is both a matrix structural molecule and an inflammatory marker was gradually increased in the A + 4 implanted pulps. At 7 days, OPN expression began to decrease [7]. For later periods of time, OPN was used

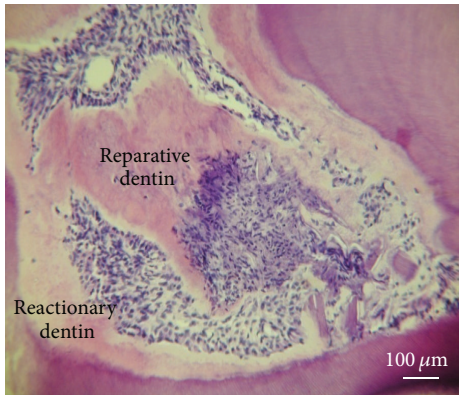



Figure 5: Reparative and reactionary dentin formation. After filling a cavity prepared in the mesial aspect of the rat's first maxillary molar, with Biodentine, a $Ca_2SiO_3$-based restorative cement, an early inflammatory reaction was stimulated. Inflammatory reaction of pulp cells producing reactionary (reduction of pulp volume) and reparative (formed in the center of the pulp volume) dentins. Hematoxylin-eosin staining. Bar = 100 $\mu$m.

exclusively as a bone cell marker because no inflammatory reaction was detected. The labeling was roughly parallel with what was observed by using a RP59 antibody, which is a marker of bone marrow cells, primitive mesenchymal cells, erythroid cells, megakaryocytes, hematopoietic precursor cells, and osteo/odontoblast progenitors. Therefore, after an initial inflammatory burst, the committed cells underwent differentiation toward an osteoblast-like phenotype. The normal dental pulp contains heterogeneous cell population, including a majority of fibroblast-like cells, but also inflammatory and immune cells [dendritic cells (DCs), histiocytes/macrophages, T-lymphocytes], and also latent or dormant pulpal stem cells (progenitors), which are mostly involved in self-renewal (Figure 5) [3]. In the intact pulp, two distinct DC populations have been identified. $CD11c^+$ are present at the pulp-dentin border, beneath occlusal fissures, whereas $F4/80^+$ DCs are almost concentrated in the perivascular region of the inner pulp and in the subodontoblastic layer. $CD11c^+$ dendritic cells express Toll-like receptors 2 and 4 and are CD205 positive. $F4/80^+$ migrate from the inner pulp, increase in size, and display CD86 expression. Anti-inflammatory agents, including steroids, interleukin-1 (IL-1) receptor antagonist, soluble tumor necrosis factor (TNF) receptor, IL-10, nitric oxide (NO), heme oxygenase-1, and regulatory T lymphocytes (Tregs), are produced in order to limit tissue damage [8]. Pulp inflammation resulting from carious lesions is characterized by a strong increase in the production of proinflammatory cytokines, including TNF-$\alpha$, IFN-$\gamma$, IL-1$\beta$, IL-6, CXCL8, and IL-18. IL-10, a cytokine that plays a central role in limiting host immune response to pathogens by promoting the development of Tregs, which are also upregulated.

Under deep caries, it is difficult to determine if the pulp is still alive or not, after bacterial invasion. Is it still possible to maintain the pulp tissue alive in the tooth? Facing an alternate possibility, the pulp should be partially or totally removed. Factors inducing inflammation may be spontaneously resolved, and in such case the pulp becomes fibrotic. It is also possible that mineralization is initiated at the periphery of the pulp, inducing the formation of a *reactionary dentin* very similar to bone or a bone-like tissue (Figure 3). In such case, *reparative dentin* may be formed, occluding the pulp exposure. It is also possible to observe diffuse mineralization or pulp stones limited in size within the pulp (Figure 4).

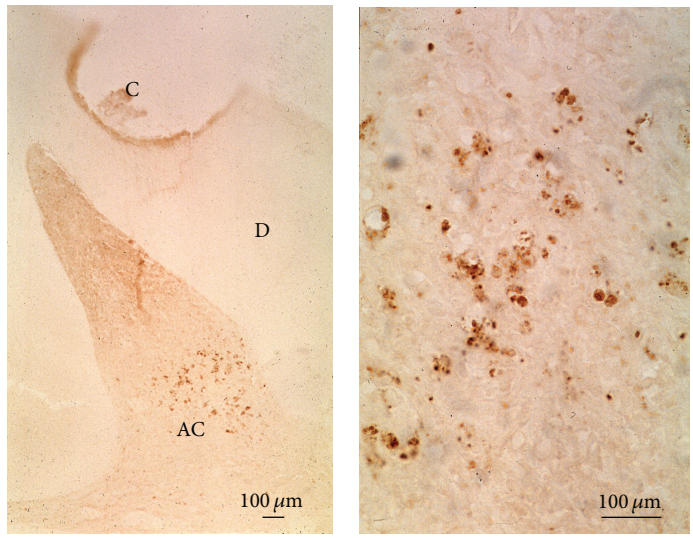
*2.2. Necrosis.* Affected pulp cells are recognized to die from two major processes: apoptosis and necrosis. It is now recognized that it is an oversimplification. Necrosis is a passive process due to the loss of protein functions or plasma membrane integrity [9]. Necrosis is caused by catastrophic toxic or traumatic events by passive cell swelling. The injury to cytoplasmic organelles, including mitochondria, leads to the rapid collapse of internal homeostasis (Figure 5). Leist et al. [10] have previously shown that intracellular energy levels are dissipated in necrosis, but not in apoptosis. Necrosis points membrane lysis, combined with the release of cellular contents that are implicated in inflammatory processes. Macrophages secrete cachectin and TNF. Bone resorption is stimulated and the phenomenon is occurring concomitantly with bone formation inhibition. Large zones of coagulation initially cause necrosis in contact with pulp connective tissue. Partially or totally infected dental pulps produce pulp calcification. A necrotic layer initiates revascularization and the construction of a dentinal bridge. Several small molecules and proteins are normally confined within cells. They are detected by specific receptors that induce a response characterized by the classical signs of inflammation at the tissue level. In areas of extensive cell death, tissue necrosis, also called coagulation necrosis, occurs.

Several other forms of cell death have been described. Cell death has been classified according to its morphological appearance (which may be apoptotic, necrotic autophagic, or associated with mitosis), functional aspects (programmed or accidental, physiological, or pathological), enzymological (with and without the involvement of nucleases or of distinct classes of proteases, such as caspases, calpains, cathepsins, and transglutaminases), or immunological characteristics (immunogenic or nonimmunogenic) [11]. Pyroptotic cell death has been described as a particular form of cell death in macrophage, induced by bacterial infection. It is accompanied by caspase-1 activation and the release of ILs. Pyroptotic cells may constitute defense mechanisms against microbial infection. The comparison between apoptosis, pyroptosis, and oncosis reveals important differences, which are shown in Table 1 [12, 13].

Caspases exist in inactive proforms in the cytosol and are activated by proteolytic cleavage by other caspases [14]. Caspases are broadly classified as apoptotic (Caspases 2, 3, 6, 7, 8, 9, and 10) or inflammatory (Caspases 1, 4, 5, and 12). Both pyroptosis and apoptosis are forms of programmed cell death that require specific caspase activity. Unlike apoptosis, pyroptosis occurs after caspase-1 activation, which does not involve in apoptotic caspases. Thus, apoptosis and pyroptosis are distinct forms of programmed cell death. Pyroptosis is

Table 1: The comparison of cellular events between apoptosis, pyroptosis, and oncosis.

| | Apoptosis | Pyroptosis | Oncosis |
|---|---|---|---|
| Initiating | Programmed | Programmed | Nonprogrammed, accidental |
| Signaling pathway | Caspase-3/6/7, DNA fragmentation | Caspase-1 DNA fragmentation | Noncaspase |
| Terminal event | Nonlytic, plasma membrane blebbing | Lytic, pore formation, and release of cytokines | Lytic |
| Effect on tissue | Noninflammatory, formation of apoptotic bodies | Inflammatory | Inflammatory |
| Cell types | All | Macrophages and DCs | All |




(a) (b)

Figure 6: Apoptotic cells formation. After treatment of rat molar with a glass ionomer, apoptosis is visualized using the TUNEL method. (a) The reaction occurs some distance away inside the mesial pulp horn of a maxillary molar. C = cavity. Apoptotic cells (AC) accumulate in the pulp. (b) Higher magnification of apoptotic cells. Apoptotic cell nuclei are fragmented. Bar = 100 $\mu$m.

viewed as a physiologically important form of cell death, which serves to eject intracellular pathogens from their replicative niche within macrophages.

*2.3. Apoptosis.* Apoptosis or programed cell death is an active process, stimulated by environmental factors [9]. Apoptosis is characterized by cell shrinkage, membrane blebbing, leading to the formation of apoptotic bodies, and, if a nucleus is present, nuclear pyknosis, chromatin condensation, and genomic fragmentation (Figure 6).

Kitamura et al. [15] have shown that the c-jun N-terminal kinase (JNK) and heat-shock proteins (HSPs) are involved in apoptosis. JNK, c-Jun, and antiapoptotic HSP are expressed in a few pulp cells. HSPs were detected in the nuclei of pulp cells and relocalized from nuclei to the cytoplasm. Investigating the dental pulp by the TUNEL method, it was possible to show that some pulp cells display apoptosis, occurring in the dental pulp, but not in the odontoblast/subodontoblast layer [16].

*2.4. Nemosis.* Fibroblasts produce a significant amount of proinflammatory cytokines and cyclooxygenase-2 (COX-2). The process is characterized as programmed necrosis-like death, which has been named "nemosis." Apoptosis is executed by caspase proteases, especially caspase-3. Although no activation of caspase-3 has been detected in nemosis, caspase inhibitors such as the pan-caspase inhibitor Z-VAD-FMK and the caspase-3 inhibitor Z-DEVD-FMK inhibited cell death by 40% and 80%, respectively [17].

Dental pulp inflammation may be a negative factor leading to pulp disruption [1]. The following three questions arise: (1) is the inflammatory reaction a prerequisite for the burst of progenitors implicated in pulp repair? (2) Does human dental pulp fibroblasts (HDPFs) formation lead in nemosis? (3) Does the adhesion between HDPFs lead to necrosis? In this context, it is well known that HDPFs express COX-2 and release prostaglandin E2 and IL-8.

Cells form spheroids were forced to cluster (also named spheroid formation). They are activated, leading to massive proinflammatory, proteolytic, and growth factor responses. Initiated by fibronectin-integrin interaction, the activation of fibroblasts ends in programming necrosis-like cell death (Figure 7).

Inflammatory reaction might be a prerequisite for the burst of progenitors implicated in pulp repair [1, 2]. Human dental pulp stem cells in culture constitute a model for

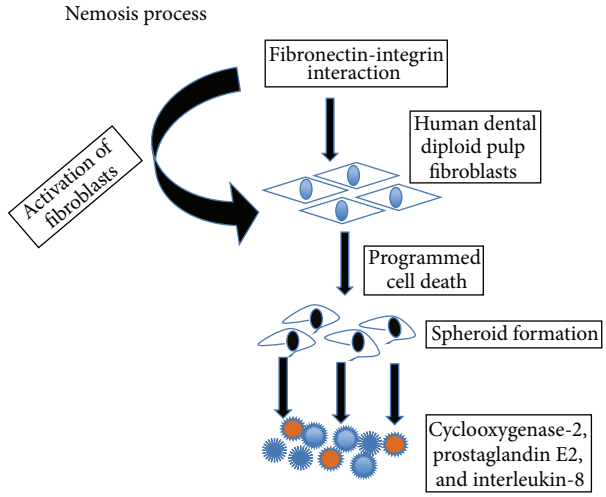



Figure 7: Illustration of nemosis process. Direct cell-cell interactions between diploid fibroblasts induce cell activation leading to programmed cell death. Nemosis of fibroblast generates large amounts of mediators of inflammation, such as prostaglandins, as well as growth factors. Factors secreted by nemotic fibroblasts also break down the extracellular matrix. Such factors include several MMPs and plasminogen activation.

*in vitro* nemosis-induced inflammation. HDPFs spheroid formation leads to necrosis. In response to nemosis, cell death is accompanied by the release of cyclooxygenase-2 and prostaglandin E2. The model supports that spheroids and interactions between fibroblasts and nemosis-targeted stem cells may contribute to treat pulp inflammation. Nemosis occurs in pulpal fibroblasts. Cell migrations may also be determined in cell sliding and migration from the root to pulp chamber [2]. Recently, it has been reported that apical stem cell niches are implicated in the sliding of apical cells from the end of the root (apex) toward the crown, where differentiation of progenitors may take place [18].

## 3. Apical Pulp Cells, Reparative Dentinogenesis, and Inflammatory Processes

*3.1. Apexogenesis and Apexification.* In young teeth, pulp vitality allows sustained root development, lengthening, and narrowing of pulp diameter. This is also named *apexogenesis* or *rhizogenesis*. If the pulp is irreversibly inflamed or necrotic, when the apex is not fully formed, procedures for the closure of apical foramina are required (*apexification*) [18, 19]. Hence, inflammatory processes in the root lead to formation three different dentins: (1) the development and lengthening of the root, (2) the formation of reactionary dentin along the root canal lumen, and (3) reparative dentin formation in the crown. In addition to the construction of the root, cellular cementum may be formed in the apical third of the root, leading to apical closure. Lateral, secondary and accessory canals contribute to the formation of complex arborescent structures. The cementum cap at the end of the root formation is apparently homogeneous and contributes to figure out how a root extension is formed. Reactionary dentin is formed, following indirect pulp capping with calcium releasing cements. Reactionary dentin is located beneath a calciotraumatic line. This newly formed dentin appears either as tubular (orthodentin structure), or as atubular with a bone-like appearance (similar to a osteodentin-like structure). It contains trapped osteocytes within osteoblasts lacunae, linked by thin canaliculi that are creating an interconnected osteocyte network. When the pulp is exposed, after capping the pulp surface is damaged by chemical injury, odontoblast-like cells differentiate beneath the scar. They polarize and are implicated in the formation of reparative dentin. This dentin, either tubular or atubular, again contributes to the formation of a dentinal bridge. The dentinal bridge is homogeneous or contains cell debris. It is also possible to have communication between the cavity and the superficial part of the pulp *via* tunnels that are containing pulp remnants. These tunnels favor bacteria communication and recontamination. More than likely, this is the reason of the failure of pulp capping after a short period of time. Tunnel and other defective structures may provide a pathway within reparative dentin for the penetration of microorganisms [6]. They are committed to develop secondary infection in pulp tissue.

Dentin phosphophoryn (DPP)/collagen composite was much superior to what resulted from calcium hydroxide regarding reparative dentin formation. Also, DPP/collagen composite displayed high ability in covering exposed pulp. It also induces the differentiation of human mesenchymal cells into odontoblast-like cells [20].

*Immunocompetent cells* are recruited in the pulp of rat after pulpotomy [21]. These cells are included within a population containing monocytes, macrophages, and stem/progenitor cells [22].

(i) *Monocytes and macrophage* originate from a common myeloid precursor in the bone marrow, expressing the colony-stimulating factor-1. The life of blood monocytes lasts just a few days before undergoing apoptosis. Monocytes can switch from a short live, undergoing apoptosis within a day to a prolonged survival during inflammation. They may go back quickly to a short live when the inflammation resolves. Macrophage life may expand up to a couple of months. Macrophages' life span has less plasticity. They may live longer and are quite resistant to apoptotic stimuli. Differentiation and inflammation determine monocyte/macrophage lifespan, by blocking the apoptotic pathway and activating many survival pathways.

There are many monocytes subpopulations, acting differently during pathogen recognition. The granulocytes are normally between 5,000 and 10,000 cells/mm$^3$ and they are composed of neutrophils (50–70%), eosinophils (2–4%), and basophils (0.5–1%). The agranulocytes are composed of lymphocytes (20–40%) and monocytes (3–8%), totally 300–700 cells/mm$^3$. The leukocytes play a fundamental role in the immune system by responding to a diverse repertoire of pathogens, including bacteria, viruses, and parasitic and

fungal infections, and also in some pathological conditions against the host cells. Monocytes and macrophages are components of the innate immune system that are responsible (1) for the recognition of the inflammatory stimuli, (2) the initiation of the inflammatory response that is characterized by the production of proinflammatory cytokines, and (3) the clearance of the pathogens allowing the resolution of inflammation. It is recognized that defined surface expression molecules characterize specific subpopulations of monocytes. They constitute the main source of resident or recruited tissue macrophages found at sites of inflammation. *Macrophages* infiltrated wound-healing sites between 1 and 28 days. Initially macrophages were described as large phagocytic cells having the capacity of "eating" wounded cells. ED1+ (CD68+) increased throughout the root pulp during an inflammatory phase. OX6+ macrophage that expresses class II MHC increases in the pulp and declines thereafter. OX6+ cells appear prior to dentin bridge formation and continue to appear during the healing stage at 14 days.

The process of differentiation from monocyte to macrophage is initiated once monocytes reach the target tissue. Monocytes can differentiate into tissue macrophages, DCs, and osteoclasts. The patrolling behavior of monocytes and macrophages is essential in the initial host response to infection. The initiation and resolution of acute and chronic inflammation are mediated by the activation of monocytes and macrophages, which are triggered by the recognition and phagocytosis of pathogens through specialized receptors.

(ii) *Stem/progenitor cells* have been identified in normal and inflamed pulp [23]. The term "stem cells" includes pluripotent cells that have an unlimited capacity to divide and are specifically adapted for permanent survival. Therefore, the next question relates to dental pulp stem cells (DPSCs) and whether they exist in the inflamed pulps (IPs). The comparison between normal and inflamed pulp cells opens in the next question. We wonder if IPs are present at higher levels in mesenchymal pulp where stem cell markers are found, such as STRO1, CD90, CD105, and CD146, or if they are present at low levels.

Leprince et al. [24] concluded that isolation and characterization of mesenchymal stem cells (MSCs) are essential for dental pulp repair. Of note, bone marrow-derived MSCs and DPSCs are probably the same, or at least of the same family. DPSCs may be recruited and be crucial for the success of regenerative endodontic procedures. The expression of specific surface antigens, for example, CD29, CD73, CD90, and CD105, may be typical for MSCs. This labeling is parallel with the absence of other surface antigens, such as CD34 or CD45. Immunocompetent cells, and especially T-lymphocytes (CD8+ T cells), differentiate into cytotoxic T cells and CD4+ T cells. They comprise also a collection of helper T cells, producing mainly Th1 and Th2. Th1 cells activate macrophages, which can produce various inflammatory mediators such as IL-1, platelet-activated factor, prostaglandins, and leukotrienes [25]. Bone marrow-derived MSCs express receptors for a large number of cytokines (e.g., IL-1, IL-4, IL-6, INF-$\gamma$, TNF-$\alpha$), and growth factors [e.g., fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), transforming growth factor-beta (TGF-$\beta$), epidermal growth factor (EGF), insulin-like growth factor (IGF), and bone morphogenetic proteins (BMPs)] and chemokines.

*3.2. Pulp Capping Hydroxide.* The mechanisms of most pulp capping hydroxide implicate that $Ca(OH)_2$ has a high pH (alkalinity) producing superficial necrosis when placed directly on exposed pulp tissue [5]. The biomaterial stimulates mineralization (the formation of dentin-like hard tissue separating the pulp from the necrotic capping agent) and has antibacterial properties. Following pulp exposure and capping, early changes include hemorrhage and moderate inflammation, resolved during the first week. The presence of calcium ions stimulates the precipitation of calcium carbonate in the wound area and thereby contributes to the initiation of mineralization. Then, the differentiation of pulp cells occurs; cells are bearing the phenotypic characteristics of odontoblast-like cells and are forming a dentin-predentin-like collagen-rich matrix. Recently, mineral trioxide aggregate (MTA) has become a popular alternative for $Ca(OH)_2$, which is composed of calcium oxide in the form of tricalcium silicate, dicalcium silicate, tricalcium aluminate, and bismuth oxide for radiopacity [26]. The MTA has a higher success rate and results in less pulpal inflammatory response and more predictable hard dentin bridge formation than $Ca(OH)_2$ [27]. MTA appears to be a suitable replacement of $Ca(OH)_2$ used for direct pulp capping; however $Ca(OH)_2$ has been considered the "gold standard" of direct pulp capping materials for several decades [28, 29].

Dentin fragments, which are displaced into the pulp during cavity preparation, are acting as initial loci for mineralization or pulp stone formation [30, 31]. Pulp inflammation, which is developed following carious lesion, is characterized by a strong increase in the expression of proinflammatory cytokines, including TNF-$\alpha$, IFN-$\gamma$, IL-1$\beta$, IL-6, CXCL8, and IL-18. Interestingly, IL-10 is a cytokine that plays a central role in limiting host immune response to pathogens and promotes the development of Tregs.

Necrosis causes slight irritation. It stimulates the defense and repair of the pulp. The observed sequence of tissue reactions exactly displayed what could be expected when a connective tissue is wounded. It starts with vascular and inflammatory cell migration, and proliferation, toward the final tissue control, eliminating the irritating agent. This process ends by the tissue repair, including migration and proliferation of mesenchymal and endothelial pulp cells, which is monitored by the formation of collagen. Human pulp in culture expresses various growth factors and cytokines, implicated in the syntheses of DNA, type I collagen, laminin, fibronectin, osteonectin/SPARC protein, and alkaline phosphatase (ALPase) [32].

The levels of type I collagen and laminin per cell were remained almost constant after culturing human pulp cells. In contrast, secreted proteins that were acidic and rich in cysteine (SPARC/osteonectin) and ALPase levels were markedly increased when the cell cultures reached confluence. Laminin and type I collagen, as well as fibronectin, stimulate the spreading of pulp cells within 1 h [33]. At

28 days, fibronectin, which is implicated in the formation and mineralization of tubular dentin, participates in the differentiation of odontoblast-like cell [33]. The addition of TGF-$\beta$ to the culture medium decreased laminin and ALPase levels, whereas it increased SPARC and fibronectin levels 3- to 10-fold [32]. Western and Northern blots showed that TGF-$\beta$ enhanced SPARC synthesis at the protein and mRNA levels. Basic FGF (bFGF) decreased type I collagen, laminin, SPARC, and ALPase levels without changing the fibronectin level [32]. PDGF selectively decreased laminin, SPARC, and ALPase levels. EGF also decreased SPARC and ALPase levels. TNF-$\alpha$ and IL-1$\beta$ decreased type I collagen and laminin levels and abolished SPARC and ALPase syntheses. bFGF and PDGF showed the greatest stimulation of [$^3$H] thymidine incorporation into DNA [32]. TGF-$\beta$, EGF, and TNF-$\alpha$ had less effect on DNA synthesis, whereas IL-1$\beta$ inhibited DNA synthesis.

Altogether, these findings demonstrated that TGF-$\beta$, bFGF, EGF, PDGF, TNF-$\alpha$, and IL-1$\beta$ have characteristically different patterns of actions on the DNA, laminin, type I collagen, fibronectin, ALPase, and SPARC syntheses by pulp cells [32]. However, there is also an alternative possibility. Noninflammatory processes may be implicated in cell recruitment, proliferation, and differentiation of pulp cells expressing phosphorylation/mineralization proteins of the extracellular matrix. There are some indications that bacteria may differently affect the odontoblasts' ability to repair the dentine barrier [34]. From the published data, there are some examples of capping effects without being associated with inflammatory processes [34].

*Emdogain Gel* initiated dentine formation, though not in a form that could constitute a solid barrier [34]. There is no evidence showing that an increased pH, and simultaneously a chemical injury, limited necrosis. The release of FGF2 delivered by a collagen sponge (noncontrolled release) or incorporated in gelatin hydrogel (controlled release) stimulates the migration and proliferation of pulp cells, followed by the invasion of vessels into dentin defects. The noncontrolled release of free FGF2 from collagen sponge induced excessive reparative dentin formation in the residual dental pulp. In contrast, controlled release of FGF2 from gelatin hydrogels induced the formation of dentin-like particles with dentin defects above exposed pulp [35].

Hindering the penetration of proinflammatory cells and/or cytokines enhanced the viability of MSCs. Using anti-inflammatory drugs and an alginate hydrogel scaffold, a molecular and cellular based investigation was reported to improve the application of hydrogels in stem cell-based therapies [36]. Along the same lines of evidences, we have implanted a light-cured hydrogel based on bovine serum albumin and glutaraldehyde within the exposed pulps of rat molars. Implanted after one week, inflammation was much more moderate compared to Dycal capping. Gradually, reparative dentin was formed, closing the pulp exposure. Dentinal bridges were formed after 3 weeks following Dycal-capping. They display tunnels and osteoblast lacunae containing osteocyte-like cells (osteodentin). The closure of the pulp exposure was not completed, and more than 4 weeks were needed to fill the gap. In contrast, the hydrogel formed more expanded and homogenous reparative dentin. There was no evidence for inflammatory processes. In both situations, active formation of the reactionary dentin layer was induced; however Hydrogel contributed more extensive dentin layer comparatively to Dycal. Hydrogel is acting as a biodegradable cavity liner, which is based on cross-linked proteinaceous material of animal/human origin [37].

The reactionary-forming dentin is not linked to pulp exposure and inflammation. There are also two different pathways of forming reparative dentin after pulp exposure. Two reparative dentins (osteodentin and orthodentin) are resulting from pulp exposure associated with inflammation or noninflammatory pulp exposure processes.

*3.3. Expression of Extracellular Matrix Proteins and Pulp Inflammation.* Phosphorylated extracellular matrix proteins (SIBLINGs) are synthesized by odontoblasts and subjacent to Hoehl's cells of dental pulp. These proteins may contribute to pulp repair and regeneration efficiently. The roles of SIBLINGs and MMPs are not diverging. SIBLINGs, as most of the phosphorylated extracellular matrix proteins, are related to the mineralization process and hence are instrumental in pulp regeneration once the inflammatory process is resolved. By contrast, MMPs are related to catalytic processes. They are acting on procollagen chains fibrillation, contributing to the cleavage of the amino and carboxyl propeptides. In addition, MMPs play role in the cleavage of dentin sialophosphoprotein (DSPP) into dentin sialoprotein and DPP. Therefore the two molecules contribute in this cascade of events reducing firstly the inflammation process, and afterwards, acting as an effective regeneration agent. In an experimental approach using germ-free and conventional laboratory rats, Kakehashi et al. [38] showed enhanced pulp repair in germ-free rats. The absence of microbial flora was the major determinant for the healing of exposed rodent pulp. They concluded that the absence of infection and inflammation was essential for tissue healing. SIBLINGs and MMPs contribute, respectively, to resolve inflammatory processes and stimulate regeneration processes.

*3.3.1. SIBLINGs and Pulp Repair. DSPP* expression is high in human dental pulp [39]. DSPP plays a role in the dentin mineralization process and is also implicated in the immune response. Leptin, an inflammation-related adipokine, and its receptor (LEPR) are expressed by human dental pulp. Immunoblot analysis and RT-PCR showed that DSPP are concentrated over the odontoblast layer; however, their presence is questionable in the central zone of the pulp. *Bone sialoprotein* produces slight inflammation following implantation to coronal pulp, and it is implicated in the formation of a homogeneous atubular dentin-like structure in the mesial part of the coronal pulp chamber one month later implantation. OP-1 (BMP7) induces the formation of osteodentin in the coronal pulp, in contrast with the radicular part of the pulp totally filled by a mineralized material after OP1 implantation [40]. According to Abd-Elmeguid et al. [41] *osteocalcin* (OCN) is a reparative molecule expressed inside the dental pulp and involved in pulpal inflammation.

OCN was positively correlated with the expression of vascular endothelial growth factor, FGF, macrophage inflammatory protein-1$\beta$, monocyte-derived chemokine, monocyte chemoattractant protein-1, IL-17, and soluble IL-2 receptor $\alpha$. It was negatively correlated with that of IL-1$\alpha$, IL-1$\beta$, IL-8, granulocyte macrophage colony-stimulating factor, and macrophage inflammatory protein-1$\alpha$. Altogether, these different properties are leading to new molecular treatment strategies. Following the role of phosphorylated proteins, Abd-Elmeguid et al. [41] has shown that dentin matrix protein-1 (DMP-1) is mostly localized in the inflammatory crown, but lacking in root pulp. DMP-1 stimulates the production of IL-6, IL-8 and has an additive effect on the release of bacterial lipopolysaccharide (LPS) on pulp cells [42].

*3.3.2. Matrix Metalloproteinases (MMPs).* MMPs are a large family of calcium-dependent zinc-containing endopeptidases, which are responsible for the tissue remodeling and degradation of the ECM, including collagens, elastins, gelatin, matrix glycoproteins, and proteoglycan. MMPs are excreted by a variety of connective tissue and proinflammatory cells including fibroblasts, osteoblasts, endothelial cells, macrophages, neutrophils, and lymphocytes.

Expression of MMP-3 was upgraded at 12 and 24 h after pulp injury, whereas MMP-2 and MMP-14 were not changed [43]. MMP-3 was localized in endothelial cells or endothelial progenitor cells in injured *in vivo* pulp. MMP-3 enhances cell proliferation, migration, and survival. It induces angiogenesis and reparative dentin formation. In isolated pulp-derived CD31−, CD146− side population cells have a highly vasculogenic potential. MMP-3 was highly expressed in CD31−, CD146− side population cells compared with CD31+, CD146− side population cells, which are without vasculogenic potential. Both MMP-9 and MMP-2 were weakly expressed in cell fractions. When CD31−, CD146− side population cells were transplanted on the amputated pulp in dogs, the transplanted cells were migrating in the vicinity of the newly formed vasculature and expressed proangiogenic factors, including MMP-3, implying trophic actions on endothelial cells [44].

*3.3.3. Other Molecules*

*Mediators.* Wnt5a is involved in inflammation regulation. Wnt5a was increased 9-fold in human dental pulp cells (HDPCs) after TNF-$\alpha$ stimulation compared with control cells [45]. HDPCs treated with Wnt5a or its supernatant increase macrophage migration (recruitment and inflammatory mediator in human pulp inflammation). Wnt5a is mitogen-activated protein kinase (MAPK) dependent and NF-$\kappa$B dependent. Wnt5a is an inflammatory mediator driving the integration of cytokines and chemokines, acting downstream of TNF-$\alpha$. Considering how pulpitis drives tissue destruction, an important step in supporting the regeneration of pulpal tissues is the attenuation of inflammation. Macrophages, key mediators of the immune response, may play a critical role in the resolution of pulpitis due to their ability to switch to a proresolution phenotype. This process can be driven by the resolvins, a family of molecules derived from fatty acids that show great promise as "therapeutic agents" [46]. Controlling inflammation facilitates dental pulp regeneration. Macrophages and neutrophils are mediators of the innate inflammatory response in the dental pulp. B and T cells of the acquired immune system infiltrate the pulp and contribute to the inflammatory response, releasing proinflammatory cytokines, IL-1$\alpha$, IL-1$\beta$ and TNF-$\alpha$, and MMPs. Two broad classes of resolvins have been characterized: the E- and the D-series, which are derived from eicosapentaenoic acid and from docosahexaenoic acid, respectively [47, 48]. Resolvins exert numerous potent anti-inflammatory effects, such as decreasing the migration and activation of neutrophils [49–51]. They inhibit the production of IL-12 by dendritic cells [52] and enhance the appearance of M2 phenotype pro-resolving macrophages [52, 53]. Resolvin E1 (RvE1) has shown efficacy in a dental context. RvE1 acts to downregulate NF-$\kappa$B through the ligand specific receptor Chem R23, which is expressed by a number of cell types, including monocytes/macrophages, neutrophils, dendritic cells, and T cells [52, 54].

*3.3.4. Vascularization.* Dental pulp is encased in dentin, which plays a role as a barrier against bacterial, chemical, and physical stimuli. When the barrier is disrupted by traumatic injury or caries, the dentin-pulp complex has a potential to repair and regenerate. Angiogenesis is essential for this pulp wound-healing process, because blood vessels play an important role in nutrition and oxygen supply, as a conduit for transport of metabolic waste, pulp homeostasis and metabolism, and stem/progenitor cell migration [55]. During pulp wound-healing process, dental pulp stem/progenitor cells migrate to the injured site from perivascular region in the pulp tissue deeper from the injured site [55]. They proliferate and differentiate into endothelial cells for angiogenesis/vasculogenesis or into odontoblasts for reparative dentin formation [56]. The angiogenic signals, such as vascular endothelial growth factor (VEGF), bFGF, and TGF-$\beta$, released from injured dental pulp cells, endothelial cells, and ECM by injury contribute to the migration of stem/progenitor cells [57–59]. In the inflamed dental pulp emphasis was put on the enlargement of blood vessels, the VEGF labeling, and pericytes, which might be at the origin of endothelial cells. The presence of enlarged vessels indicates (1) an increased tissue fluid pressure; (2) a greater outward flow of dentinal fluid; and thus (3) an increased pain after dentinal stimulation [60]. Neoangiogenesis is a requirement for regeneration and healing, highly controlled by the microenvironment [61]. Precise mechanism for migrating stem/progenitor cells and angiogenesis/vasculogenesis during pulp wound-healing process, however, still remains unclear.

## 4. Conclusion

Different phases of pulp inflammation have been identified, associated with moderate inflammation, necrosis including pyroptosis, apoptosis, and nemosis. For many years

the importance of inflammation in pulpal healing has been underestimated, considered only as an undesirable effect. There are now evidences that inflammation is a prerequisite for tissue healing and pulp regeneration. Immunocompetent cells (monocytes, macrophages, and stem/progenitor cells) are recruited. Cells slide along the root and migrate toward the crown. Due to the high alkalinity of the capping agent, mineralization is initiated and becomes thicker. Due to inflammatory processes, pulp cells proliferate and display increased number and size. Their phenotype is modified, and they become odontoblast-like cells producing collagen, ALPase and SPARC/osteonectin.

Molecules of the SIBLING family, MMPs, mediators, and scaffolds are also implicated in the formation of a reparative dentinal bridge. These molecules are implicated in the direct formation of osteo/orthodentin, occluding the pulp exposure. There is also an alternative possibility that noninflammatory processes contribute to produce reparative dentin. This suggests that there is occurrence of different reparative pathways after the pulp exposure. Dentins are formed as reactionary dentin, an accumulation occurring at the surface of the dental pulp, along the periphery of the pulp chamber, or as reparative dentin creating a dentinal bridge occluding partially or totally a pulp exposure.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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*Review Article*

# Dental Pulp Defence and Repair Mechanisms in Dental Caries

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Dental caries is a chronic infectious disease resulting from the penetration of oral bacteria into the enamel and dentin. Microorganisms subsequently trigger inflammatory responses in the dental pulp. These events can lead to pulp healing if the infection is not too severe following the removal of diseased enamel and dentin tissues and clinical restoration of the tooth. However, chronic inflammation often persists in the pulp despite treatment, inducing permanent loss of normal tissue and reducing innate repair capacities. For complete tooth healing the formation of a reactionary/reparative dentin barrier to distance and protect the pulp from infectious agents and restorative materials is required. Clinical and *in vitro* experimental data clearly indicate that dentin barrier formation only occurs when pulp inflammation and infection are minimised, thus enabling reestablishment of tissue homeostasis and health. Therefore, promoting the resolution of pulp inflammation may provide a valuable therapeutic opportunity to ensure the sustainability of dental treatments. This paper focusses on key cellular and molecular mechanisms involved in pulp responses to bacteria and in the pulpal transition between caries-induced inflammation and dentinogenic-based repair. We report, using selected examples, different strategies potentially used by odontoblasts and specialized immune cells to combat dentin-invading bacteria *in vivo*.

## 1. Odontoblasts in the Dental Pulp's Defence against Caries

The crowns of erupted human teeth are covered by symbiotic microbial communities, mainly composed of Gram-positive saprophytic bacteria which are normally harmless to the tooth. These communities adhere as biofilms to the highly mineralized enamel that constitutes a barrier which is impermeable to microorganisms and protects the underlying mineralized dentin and the loose connective tissue situated at the centre of the tooth, the dental pulp. However, when placed in a sugar-rich environment, specific bacterial populations from these communities release acids that progressively demineralize enamel [1, 2]. This leads to the appearance of a carious lesion characterized by a cavity within which "cariogenic" bacteria proliferate and release additional acids that progressively deepen the lesion. When the enamel barrier is disrupted, dentin becomes degraded by Gram-positive bacteria, including streptococci, lactobacilli, and actinomyces that largely dominate the dentin caries microflora [3]. The proliferation and metabolic activity of these microorganisms lead to the release of bacterial components into dentinal tubules and their diffusion towards the peripheral pulp. Dentin demineralization may also enable the release of bioactive molecules from the dentin matrix [4]. Recognition of bacterial components by host cells at the dentin-pulp interface triggers host protective events including antibacterial, immune, and inflammatory responses. These events may

eliminate early stage bacterial infection and block the route of its progression when accompanied by dentin formation at the pulp-dentin interface. Unchecked, bacterial invasion results in irreversible chronic pulp inflammation, most often after a long phase of chronic inflammation. Subsequently, pulp necrosis, infection of the root canal system, and periapical disease may occur [3, 5]. Pulp inflammation, also called "pulpitis," generally dampens after microorganism removal by the dental practitioner and neutralization of intratubular diffusing components by the pulp immune system, both decreasing the production of proinflammatory mediators [6]. However, when the caries lesion is close to the dentin-pulp interface, pulpal inflammation does not resolve completely after dental treatment and may become low-grade and chronic in nature. This chronic inflammation is responsible, as in other connective tissues, for the permanent loss of normal tissue function and the reduction of defence capacities to future injuries. On occasions, rapid cessation of inflammation enables complete pulp healing with the formation of a barrier of reactionary dentin by the original surviving odontoblasts and/or reparative dentin by newly differentiated odontoblast-like cells in animal models [7]. Dentin neoformation protects the underlying pulp from the dentin infection and the crown filling biomaterial, thus reducing the risk of permanent irritation by external bacterial or chemical agents. It is reasonable to speculate that rapid reactionary/reparative dentin formation is initiated, the quicker pulp healing occurs, and health is reestablished. So, from a clinical point of view, it appears crucial to identify molecular and cellular agents able to dampen immune/inflammatory events within the dental pulp and promote rapid return to tissue homeostasis and health once the bacterial infection is resolved [2, 8–10]. Such agents should help to prevent the evolution of the pulp inflammation towards becoming chronic in nature. To identify these agents, it is important to gain an in-depth knowledge of the events that initiate and control the early steps of human pulp antibacterial defence and dentinogenesis-based reparative mechanisms in caries-affected human teeth. This paper focusses on key cellular and molecular mechanisms involved in pulp responses to bacteria and in the pulpal transition between caries-induced inflammation and dentinogenic-based repair. We report, using selected examples, different strategies potentially used by odontoblasts and specialized immune cells to combat dentin-invading bacteria *in vivo*.

Odontoblasts are the first pulpal cells encountered by dentin-invading pathogens and their released products owing to both their specific localization at the pulp-dentin interface and the embedding of their long cellular processes in dentin tubules. We and others have therefore hypothesized that, in the tooth, they represent the first biologically active line of defence for the host, fulfilling the role devoted elsewhere in the body to skin and mucosal epithelial cells [12, 13]. Odontoblasts may thus be involved in combatting bacterial invasion and activating innate and adaptive aspects of dental pulp immunity. Both these events can only be activated following pathogen recognition by pulp cells. In a general way, such recognition occurs through the detection ("sensing") of molecular structures shared by pathogens and that are essential for microorganism survival. These structures are termed Pathogen-Associated Molecular Patterns (PAMPs) and are sensed by a limited number of so-called Pattern-Recognition Receptors (PRRs). One important class of PRRs is represented by the Toll-like receptor (TLR) family that is crucial for the triggering of the effector phase of the innate immune response [14–16]. TLR2 and TLR4, which are involved in Gram-positive and Gram-negative bacterial sensing, respectively, have been previously detected in the odontoblast cell membrane in healthy pulp, indicating that odontoblasts are equipped to recognize these pathogens when they diffuse through dentin tubules during the carious infection [13, 17]. TLR2 has been shown to be upregulated in odontoblasts beneath caries lesions compared with odontoblasts beneath healthy dentin [2], suggesting that these cells are not only adapted to the recognition of Gram-positive bacteria but that they are also able to amplify their response to these pathogens.

One major consequence of TLR activation is upregulation of innate immunity effectors, including antimicrobial agents and proinflammatory cytokines and chemokines that recruit and activate tissue resident and blood borne immune/inflammatory cells [18, 19]. Odontoblasts have been found to produce several antibacterial agents, among which beta-defensins and nitric oxide have received particular attention. Beta-defensins (BDs) are cationic, broad-spectrum antimicrobial peptides that kill microorganisms by forming channel-like micropores that disrupt membrane integrity and induce leakage of the cell content [20–23]. They are mainly produced by epithelial and immune cells to protect skin and internal mucosae from pathogen invasion. Whereas BD-1 is generally constitutively expressed, BD-2, BD-3, and BD-4 are induced by microorganisms that come into contact with host cells. Several *in vitro* studies have reported that BDs might also be involved in the pulpal defence against caries-related microorganisms. Indeed, BD-2 was shown to possess antibacterial activity against *S. mutans* and *L. casei* [24–26] and BD-3 exhibited antibacterial activity against mature biofilms containing *Actinomyces naeslundii*, *Lactobacillus salivarius*, *Streptococcus mutans*, and *Enterococcus faecalis* [27]. A proinflammatory role was also proposed for BD-2, which upregulates interleukin (IL-) 6 and as Chemokine [C-X-C Motif] Ligand 8 (CXCL8, also known as IL-8) in odontoblast-like cells *in vitro* [28]. A positive feedback mechanism could exist between inflammatory cytokines and BD-2, the expression of which was found to be stimulated by IL-1$\alpha$ and tumor necrosis factor (TNF-) $\alpha$ in cultured human dental pulp cells [29, 30]. The proinflammatory effect of BD-2 could be augmented by the fact that it chemoattracts immature antigen-presenting dendritic cells (DCs), macrophages, CD4+ memory T cells, and natural killer (NK) cells by binding to cell surface chemokine receptors [22]. *In vitro*, odontoblast BD-2 gene expression was not modified by TLR2 activation in a tooth organ culture model, whereas BD-1 and BD-3 genes were downregulated [13]. BD-2 gene expression was upregulated upon TLR4 activation, which suggests that BDs are differentially produced by odontoblasts to combat Gram-positive and Gram-negative bacteria. *In vivo* studies have revealed that odontoblasts in healthy pulp synthesize

BD-1 and, to a lesser extent, BD-2 [31, 32]. Constitutive expression of low levels of BDs in the odontoblast layer might be necessary to destroy individual or very small groups of oral early stage bacterial invaders which enter the tooth through tiny, clinically undetectable lesions such as enamel cracks, before these bacteria engage with the pulpal immune system. Discrepancies exist between reports regarding the regulation of BDs in inflamed dental pulp. Indeed, BD-1 and BD-2 were first reported to be decreased during irreversible pulpitis [28], whereas, in a more recent study, BD-1 and BD-4 were found to be increased in inflamed pulps compared with healthy ones; the expression of BD-2 and BD-3 however remained constant [32]. Differences in the inflammatory status between pulp samples (reversible versus irreversible inflammation) may be responsible for these discrepancies. It remains unclear as to whether BDs are present in the bacteria-challenged inflamed pulp at levels that enable them to play a major role in the tissue defence against dentin-invading bacteria. Further studies are needed to investigate the antibacterial activity of BDs produced at *in vivo* relevant concentrations by odontoblasts challenged with caries-related microorganisms. Another important antimicrobial agent produced by odontoblasts challenged with microbial components is nitric oxide (NO). NO is a potent antibacterial, highly diffusible free radical produced from L-arginine through oxidation by NO synthases (NOS), of which there are 3 isoforms: NOS1 (neuronal NOS) and NOS3 (endothelial NOS), that are constitutively expressed in most healthy tissues, and NOS2 (inducible NOS), generally absent from healthy tissues and induced in particular in tissues challenged by microorganisms. NOS1 and NOS3 are constitutively expressed in physiological conditions by many cells and produce very low, picomolar to nanomolar range NO concentrations within seconds or minutes. NOS2 is mostly involved in host defence by producing high, micromolar range amounts of NO for sustained periods of time (hours to days) [33–39]. NOS2 is not, or only moderately, expressed in healthy human dental pulps and was found to be rapidly upregulated in inflamed pulps [40–44]. Furthermore, NOS2 activation was shown to promote the accumulation of neutrophils and macrophages in experimentally inflamed rat incisor pulps [42, 43]. CXCL8 might also be involved in this process since NO has been shown to stimulate the production of this chemokine in human pulp cells *in vitro* [45]. Human odontoblasts in the inflamed dental pulp showed a marked immunoreactivity for 3-nitrotyrosine (a biomarker for NO-derived peroxynitrite), suggesting that these cells release NO upon NOS2 activation [44]. Indeed, NO release might constitute an important defence mechanism against *Streptococcus mutans* as the growth of these microorganisms has been shown to be inhibited by NO *in vitro* [46]. Accordingly, NO produced at high concentration by NOS2 in the inflamed pulp might be used by odontoblasts as a weapon to combat cariogenic bacteria. We have recently presented evidence that odontoblasts differentiated *in vitro* strongly amplify their NOS2 synthesis and NO production upon TLR2 activation. The NO produced was found to inhibit the growth of *Streptococcus mutans*, thus suggesting the role of this odontoblast-derived molecule in the limitation of the intradentinal progression of caries-related microorganisms [47].

Numerous *in vitro* studies have also shown that odontoblasts produce inflammatory cytokines and chemokines when challenged by PAMPs from Gram-positive bacteria [12, 13]. In particular, odontoblasts differentiated *in vitro* were found to be responsive to lipoteichoic acid (LTA), a Gram-positive bacteria wall component recognized at the cell surface through TLR2. Engagement of odontoblast TLR2 by LTA upregulated TLR2 itself and NOD2, a cytosolic PRR, which led to nuclear factor-$\kappa$B (NF-$\kappa$B) and p38 mitogen-activated protein kinase (MAPK) signalling activation, dentinogenesis inhibition, and production of the proinflammatory chemokines Chemokine [C-C Motif] Ligand 2 (CCL2), CXCL1, CXCL2, CXCL8, and CXCL10 [2, 12, 48–51]. Chemokine production by odontoblasts following bacterial challenge might attract immune cells into the odontoblast layer beneath the carious lesion [52]. Indeed, when dentin is being demineralised by caries, immature DCs accumulate at an early stage at the dentin-pulp interface in a strategic location to capture foreign antigens. A progressive and sequential accumulation of T cells (= T lymphocytes), macrophages, neutrophils, and B cells (= B lymphocytes) then occurs in the pulp, concomitantly with the deepening of the dentin lesion, the increase of the bacterial insult, and the development of the pulp inflammatory process [6, 53]. Thus, it is likely that odontoblasts are able to attract some, if not all, of these immune cell populations at the pulp-dentin interface to neutralize bacterial by-products that reach the pulpal end of the dentin tubules. By using culture supernatants of odontoblast-like cells stimulated with TLR2 agonists, we demonstrated that odontoblasts produced chemokines able to recruit immature DCs [12, 48]. CCL2, strongly expressed in odontoblasts beneath dentin carious lesions, may be involved in this process since it is a key element in the recruitment of circulating blood dendritic cells. Odontoblast-derived CXCL1, CXCL2, and CXCL8, which are known to attract neutrophils, and CXCL10, known to attract T cells, could be involved in the accumulation of other populations of immune cells at the dentin-pulp interface. However, to our knowledge, no direct evidence for a role of odontoblast-derived chemokines in these processes has been reported so far.

IL-6 is a pleiotropic cytokine produced by a variety of immune and nonimmune cells that regulates many aspects of the local immune response [54]. It is strongly upregulated in bacteria-challenged inflamed pulps *in vivo* and in odontoblasts *in vitro* upon TLR2 engagement [49, 55]. IL-6 is notably critical to the differentiation and regulation of T helper (Th)2, Th17, and T regulatory (Treg) phenotypes, and it promotes the secretion of acute-phase proteins including lipopolysaccharide-binding protein [19]. All these functions might be undertaken in inflamed pulps by IL-6. Since it also increases vascular permeability, IL-6 might also be involved in the formation of oedema induced by the progressive intradentinal penetration of Gram-positive oral bacteria [49].

IL-10 is an immunosuppressive cytokine produced by many immune and nonimmune cells which modulate immune responses to microbial antigens in order to prevent excessive or unnecessary inflammation. It acts in particular by decreasing the production of the proinflammatory

cytokines IL-6 and CXCL8, thereby suppressing inflammation-associated immune responses and limiting damage to the host [56]. It also inhibits Th1 and Th2 immune responses but promotes the differentiation of regulatory T cells which control excessive immune responses in part by producing IL-10, which provides a positive regulatory loop for IL-10 induction [57, 58]. We found that IL-10 is upregulated in bacteria-challenged inflamed pulps *in vivo* [49] where it might help limit the spread of pulp inflammation which is initially restricted to the dentin-pulp interface beneath early dentin caries lesions [59]. IL-10 was upregulated in odontoblast-like cells *in vitro* upon TLR2 engagement, suggesting that odontoblasts are capable not only of initiating the pulp immune and inflammatory response to dentin-invading bacteria, but also of limiting its intensity [49].

Recently, we have studied the role of lipopolysaccharide-binding protein (LBP), an acute-phase protein known to attenuate proinflammatory cytokine production by activated macrophages. LBP has been shown to prevent the binding to host cells of several bacterial cell wall components including lipopolysaccharides, lipoteichoic acids, lipopeptides, and peptidoglycan [60]. It was also found to transfer lipopolysaccharides to high-density lipoproteins in the plasma for neutralization [61]. We recently detected LBP synthesis and accumulation in bacteria-challenged inflamed pulp, whereas this protein was not found in healthy pulp. *In vitro*, LBP was upregulated by Pam2CSK4 (a diacylated lipopeptide synthetic analog that binds specifically TLR2) in odontoblasts differentiated *in vitro*. It also decreased TLR2 activation and attenuated proinflammatory cytokine synthesis ([62], unpublished results). This molecule might be involved in the neutralization of bacterial components that gain access to the pulp, thus limiting activation of the pulp immune cells and the associated inflammatory response to dentin-invading bacteria [8].

In summary, numerous studies performed over the last decade have shown that odontoblasts are able to detect oral microorganisms that invade mineralized dental tissues from the oral cavity. They mobilize themselves against this threat by building their own antibacterial arsenal (defensins, nitric oxide) and by sending molecular messengers (chemokines, cytokines) to the neighbouring pulp to alert immune cells able to mount responses to microorganisms (Figure 1). However, the majority of these studies have been performed *in vitro* and currently minimal information is available about the nature and role of antibacterial and immune effectors in caries-affected teeth *in vivo*. Additional experiments are therefore warranted to further characterize the molecular effectors and regulators of human dental pulp immunity and determine their therapeutic potential to promote the recovery of dental pulp homeostasis and health.

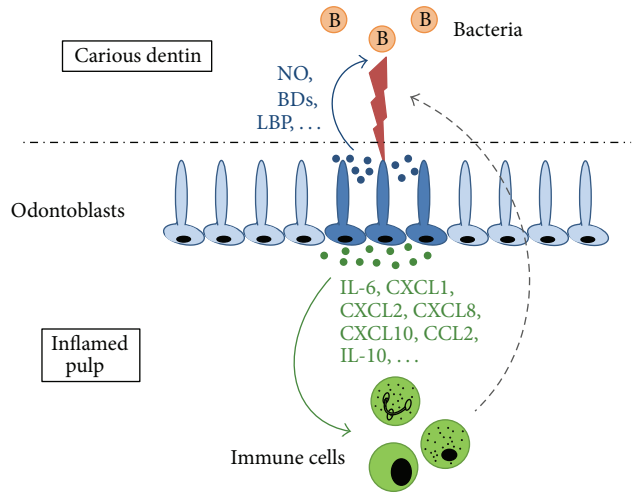



Figure 1: Two key aspects of the odontoblast defence against dentin-invading bacteria. Bacteria (B) present in the carious dentinal lesion release pathogenic components that activate (blue arrow) odontoblasts (dark blue) adjacent to the lesion, triggering the production of antibacterial molecules (blue dots). These molecules diffuse through dentin tubules in an attempt to destroy the invading microorganisms (NO, BDs) or considerably decrease their pathogenicity (LBP). In parallel, proinflammatory and immunomodulatory mediators (green dots), including IL-6, IL-10, CXCL1, CXCL2, CXCL8 (IL-8), CXCL10, and CCL2, are secreted by odontoblasts at the opposite cell pole and diffuse into the subodontoblast pulp area (green arrow) where they activate and mobilize various populations of immune cells (as described in the main text body) enabling the immunosurveillance of the tissue. Immune cells then migrate (dotted grey arrow) towards the pulp-dentin interface beneath the lesion to combat the bacteria and coordinate the immune defense response.

## 2. Response of Pulp Immune Cells to Tooth-Invading Pathogens

As stated above, eliminating the decayed mineralized tissues containing microbial agents can result in decreased pulpal inflammation, promotion of tissue healing, and restoration of the normal biological functions of the pulp. Like peripheral organs and tissues such as skin, gastrointestinal tract, and lungs, healthy dental pulp contains sentinel leukocytes, which are able to biologically sample and respond to the local environment, including macrophages, DCs, and T cells [52, 53, 63, 64]. Fluorescence-activated cell sorting (FACS) analysis of enzymatically digested whole pulp tissue revealed that leukocytes represent ~1% of the total cell population in nonerupted human third molars [10]. Leukocytes in healthy tissue undertake immunosurveillance, that is, continuous sampling of their environment to sense microorganisms invading into the body. Their numbers significantly increase when pathogens are detected, due to the elevation of the inflammatory process. This inflammation is part of the normal protective immune response of the host to tissue infection and during this response, leukocytes from the circulatory system are triggered to adhere to endothelial cells lining blood vessels prior to them migrating out of the blood vessel to the site of infection. Neutrophils are initially recruited to the inflamed tissue to engulf and destroy invading microorganisms; subsequently this response is followed by monocytes which also differentiate into macrophages. In teeth, neutrophils and macrophages progressively infiltrate the pulp tissue as the carious disease progresses [4, 6, 9, 53, 65–67].

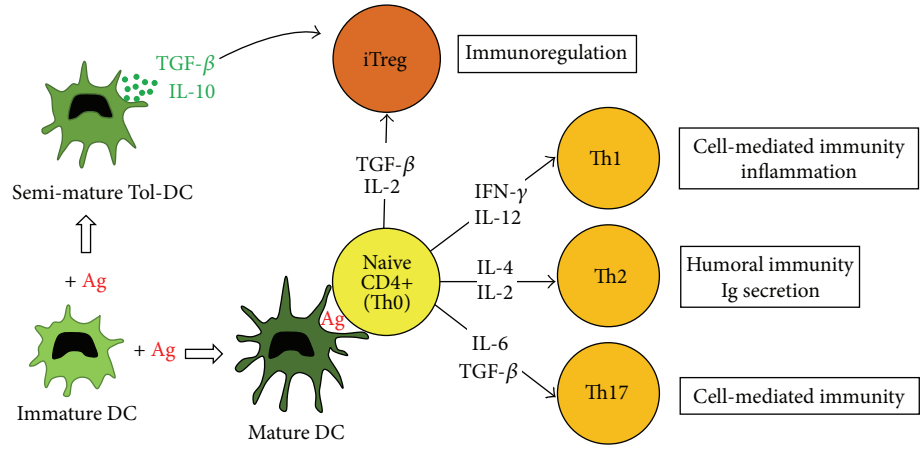



FIGURE 2: The putative role of dendritic cells (DCs) in the regulation of T helper (Th) and induced regulatory T (iTreg) cell differentiation. Upon encountering antigens (Ag), immature DCs usually become mature DCs which present antigens to naive CD4+ (Th0) cells. Upon antigen recognition, Th0 cells clonally expand and can differentiate into various subsets of effector cells (Th1, Th2, or Th17) or into iTreg cells depending on the cytokines present in their environment. Alternatively, immature DCs can mature only partially to become Tolerogenic-DCs (Tol-DCs) which can directly induce iTreg cell differentiation through TGF-$\beta$ and IL-10 secretion. IL, interleukin; IFN, interferon; TGF, transforming growth factor; Ig, immunoglobulin.

Macrophages are able to phagocytose bacteria and activate T cells triggering an adaptive immune response which occurs in association with DCs. In the pulp, DCs are initially present in an immature state and are attracted by odontoblast-derived chemokines to the site of infection, where they capture bacterial antigens diffusing through dentin tubules towards the pulp [6, 12, 48, 53]. Antigen uptake triggers the activation and progressive maturation of DCs, and they subsequently migrate to regional lymph nodes where they present antigens to, and activate, naive CD4+ T cells (also called Th0 cells). Activated DCs secrete a range of cytokines that influence both innate and adaptive immune responses, and they are considered key regulators of the tissue's defence against infection. Naive CD4+ T cells, when activated, can differentiate into effector CD4+ T helper cells or induced regulatory T (iTreg) cells [68]. Furthermore effector CD4+ T cells are classically assigned to Th1, Th2, or Th17 subsets and undertake specific functions in the immune response including regulation of cell-mediated immunity, inflammation, and protection against intracellular pathogens. Th1 cells are generated by IL-12 and interferon (IFN-) $\gamma$ exposure and they secrete IFN-$\gamma$, IL-2, and TNF-$\alpha$. Naive CD4+ T cells differentiate into Th2 cells following exposure to IL-4 and IL-2. Th2 cells produce IL-4, IL-5, IL-6, IL-10, IL-13, and IL-14; they regulate humoral (immunoglobulin-mediated) immunity and are involved in protection against extracellular pathogens. The Th17 lineage pathway provides a unique mechanism for protection against bacterial and fungal pathogens through the production and induction of inflammatory cytokines and the recruitment of neutrophils. Th17 cells are induced to differentiate from naïve CD4+ T cells mainly by transforming growth factor (TGF-) $\beta$ and IL-6 [69] (Figure 2). We have previously provided precise quantification of T cells in healthy human dental pulp, enabling a better understanding of the initial capacity of the pulp to detect and combat pathogens. Our data demonstrated that cytotoxic CD8+ T cells represented ~21% total leukocytes, and CD4+ T cells were ~11%, with DCs ~4% of the leukocyte population. We observed that progressive and sequential accumulation of CD4+ and CD8+ T cells was observed in inflamed pulp which occurred in parallel with the deepening of the dentin lesion [4, 53, 67]. Elucidating the exact mechanisms that regulate Th1, Th2, or Th17 responses is essential to more comprehensively understand pulp pathogenesis; however to date no data are available regarding the subsets of T cells involved in these mechanisms. Thus far only one study has reported pulp regeneration in a mild irreversible pulpitis model after inhibition of IL-6 secretion by matrix metalloproteinase (MMP-) 3. The authors proposed that the control of IL-6 activities by MMP-3 could thus decrease the Th2 response and Th17 cell induction [70]. NK cells are also a well-known arm of the innate immune system. They are reported to exhibit features characteristic of the adaptive immune response and they have recently been identified in healthy rat molar pulps [71]. We have now found that NK cells represented ~2.5% of leukocytes in human healthy pulp [10]. In addition, a subset of T cells known as natural killer T (NKT) cells has been detected in healthy rat pulp [71] and these cells are known to play a major role in the development of Th1 versus Th2 immune responses [72]. Finally, a relatively small number of B cells are present in healthy pulp tissue and their numbers significantly increase during pulpitis and caries progression [10, 73]. Immunohistochemical analysis of inflamed pulp demonstrated that B cell-derived IgG1, rather than IgG2, is the dominant subclass of immunoglobulin followed by IgA and IgE [4, 65]. During human dental root resorption, B cells form clusters in the pulp of deciduous teeth [74] and their role may be to modulate DC functions [75].

In order to avoid irreversible damage to the pulp tissue, the complex immune responses must be controlled to enable

pathogen destruction without causing damage to the host. Regulatory cells play a major role in this process [76]. In particular, subpopulations of immature DCs, called Tol-DCs, are resistant to maturation and are implicated in the regulation of the immune response [77]. They induce central and peripheral tolerance through different mechanisms including T cell depletion or anergy, induced Treg cell differentiation from naive CD4+ T cells, and production of a variety of immunomodulatory mediators such as PD-L1, PD-L2, heme oxygenase-1 (HO-1), HLA-G, galectin-1, DC-SIGN, IL-10, TGF-$\beta$, indoleamine 2,3-dioxygenase, IL-27, and NO [78, 79]. Naive CD4+ T cells differentiate into induced Treg cells (iTregs) following exposure to TGF-$\beta$ and IL-2. They express CD4, CD25, and FoxP3 and secrete TGF-$\beta$ and IL-35 that inhibit the effector T cell response. Among the iTreg population, Tr1 cells secrete a large quantity of IL-10 and TGF-$\beta$ which suppress Th responses [80]. Relatively large numbers of iTregs have been detected in intensely inflamed human pulps [81]. FACS analysis, using healthy human molars, resulted in the detection of iTregs identified by the phenotype CD45+CD3+CD4+CD127low CD25+ and Foxp3+. There is also now evidence for the presence of a specific subset of DCs expressing HO-1 in healthy human pulp [10]. DCs expressing HO-1 have immunoregulatory properties, as this enzyme protects cells against inflammatory and oxidative stress [82]. Furthermore, myeloid derived suppressor cells (MDSCs) have been identified in healthy pulp and they constitute a heterogeneous population of cells with a remarkable ability to regulate immune responses [83–85]. Notably MDSCs expanded by exposure to bacterial components, such as lipopolysaccharide (LPS), regulate alloreactive T cells via HO-1 and IL-10 secretion [86]. Together, these results indicate that healthy dental pulp is equipped for limiting or fine-tuning innate and adaptive responses even in the absence of pathogens.

In summary, healthy dental pulp contains resident immune cells and is thus initially well equipped to detect and mount effective immune responses against invading pathogens. Recruitment of circulating immune cells into the pulp tissue during the inflammatory process reinforces its defence potential. In particular, it has recently been reported that the range of resident leukocytes is much wider in healthy pulp than previously understood and includes several populations of cells with immunoregulatory properties. These data indicate that the immune and inflammatory dental pulp response to pathogens is extremely complex. Additional studies are therefore warranted to understand how such a response can be controlled to promote tissue healing after pathogen removal by the dental practitioner.

## 3. Inflammation-Regeneration Interplay in the Dentin-Pulp Complex

Clearly, defence and reparative responses within the tooth are inextricably linked. During carious disease, which damages the tooth structure, the host aims to both fight the infection, via its immune-inflammatory response, and "wall off" and restore the tooth structure, via its dentinogenic responses.

Notably, the regenerative mechanisms within the dental tissues are underpinned and informed by developmental processes. Following a series of molecular and cellular signalling events which occur between the developmental epithelium and mesenchymal tissue, odontoblasts differentiate from progenitor cells bordering the dental papilla. In brief, they take on a polarised columnar form and secrete predentin and further signalling leads to cells of the inner enamel epithelium, which are in contact with the predentin, differentiating into polarised columnar ameloblasts, which subsequently synthesise the enamel. The predentin is converted to dentin and further cycles of predentin secretion and mineralisation result in the odontoblasts receding from the dentinoenamel junction towards the pulp core. As the dentin structure of the tooth develops, the odontoblasts leave their cellular processes extended within the dentinal tubules. A multitude of genes have been identified as being active during tooth development and morphogenesis, which indicates the complexity of the process [87]. Indeed, many of the growth factors involved in signaling the dentinogenic process subsequently become fossilised within the dentin as they are secreted by the odontoblast during development. Notably, their later release from the dentin during disease is understood to regulate both regenerative and defensive responses within the tooth and is discussed in more detail below.

Whilst primary dentinogenesis occurs at a rate of ~4 $\mu$m/day of dentin deposition during tooth development, secondary dentinogenesis decreases to a rate of ~0.4 $\mu$m/day following root formation and continues to occur throughout the life of the tooth. Tertiary dentinogenesis however describes the process of hard tissue repair and regeneration in the dentin-pulp complex, which is the tooth's natural wound healing response. With milder dental injury, such as early stage dental caries, primary odontoblasts become reinvigorated to secrete a reactionary dentin which is tubular and continuous with the primary and secondary dentin structures. However, in response to injury of a greater intensity, such as a rapidly progressing carious lesion, the primary odontoblasts die beneath the lesion [88, 89]. While it is not entirely clear what causes this odontoblast cell death, it is hypothesized that bacterial toxins, components released from the demineralised dentin or even local generation of high levels of proinflammatory mediators, signal this event. Subsequently, however, if conditions become conducive (e.g., if the carious infection is controlled or arrested), stem/progenitor cells within the pulp are signalled to home to the site of injury and to differentiate into odontoblast-like cells. These cells deposit a tertiary reparative dentin matrix, reportedly at a similar rate to that of primary dentinogenesis, and this clinically results in dentin bridge formation. The new hard tissue deposited walls off the dental injury and the infecting bacteria, protecting the underlying soft tissues, and partially restores tooth structure [90]. Clearly the relative complexity of these two tertiary dentinogenic processes differs, with reactionary dentinogenesis being comparatively simple and requiring only upregulation of existing odontoblast activity, whereas reparative dentinogenesis is more complex and involves recruitment, differentiation, and upregulation of dentin synthetic and secretory activity. Notably, it is

understood that tertiary dentin deposition rates somewhat recapitulate those in development with dentin. Tertiary dentinogenic events are also understood to be signalled by bioactive molecules, similar to those present during tooth development. Some of these molecules may arise from the dentin when it is demineralised by bacterial acids as a variety of growth factors and other signalling molecules are sequestrated within the dentin during its deposition and formation [90–92]. The breakdown and release of signalling molecules from the dentin provide a means by which the tooth can detect tissue damage and subsequently rapidly respond. Indeed, an array of molecules are bound within dentin and are known to be released from their inactive state by carious bacterial acids, as well as restorative materials, such as calcium hydroxide, which are known to stimulate dentin bridge formation following clinical application. Furthermore a variety of molecules which in general are regarded as inflammatory mediators are also implicated in signalling repair responses. Clearly, it is likely that a fine balance exists between their levels and temporal and contextual profiles, which subsequently regulates the effects of these molecules on dental cells and tissues. These signalling aspects are further discussed below in more detail.

The carious infection, if unchecked, will progress through the dental hard tissues and into the soft pulpal core. In general, markers of the inflammation also subsequently increase including levels of cytokines and the immune cell infiltrate [64, 73, 93]. Indeed, the increased levels of cytokines have a range of regulatory functions including lymphocyte recruitment, extravasation, activation, differentiation, and antibody production. The roles of the cytokines, IL-1$\alpha$, IL1-$\beta$, and TNF-$\alpha$, are particularly well characterized in orchestrating the immune response in the pulp in response to carious and deeper associated periapical infections [93–100]. Initially, as has been discussed, resident pulp cells, including odontoblasts, will increase their expression of these molecules; however, a range of immune cells recruited to the lesion in response to infection will further add to the molecular milieu. Furthermore, components of dentin released by carious bacterial acids during the demineralization process have also been demonstrated to contribute to the levels of inflammatory mediators [101]. Notably, many other cytokines including IL-4, IL-6, IL-8, and IL-10 have been shown to be increased in pulp tissue, which is affected by carious disease [102–104]. It is a range of these potent cytokine signaling molecules which generates the chemotactic gradients leading to recruitment and activation of the immune cells described above and can subsequently lead to the chronic cycle of inflammation present within the tooth [105, 106].

Notably, the cytokine IL-8 is constitutively expressed by odontoblasts, likely in anticipation of disease events, and its levels can be significantly upregulated both by bacterial components (e.g., LPS via TLR signaling mechanisms) and by IL-1$\beta$ and TNF-$\alpha$ in a range of cell types [107]. IL-8 is particularly important in the recruitment and activation of neutrophils, which are generally one of the first immune cell types present at the site of infectious disease (as described in detail above). Interestingly, we have reported elevated levels at both the transcript and protein levels for a range of proinflammatory mediators, including S100 proteins, in carious diseased pulpal tissue compared with healthy pulpal tissue [66, 93].

While local release and accumulation of proinflammatory mediators occur in response to the progressing carious infection, data now indicate that bacterial acid-driven dentin demineralization likely adds to the complex cocktail of signaling molecules present within the diseased dental tissue [66]. As we are aware that odontoblasts basally express certain cytokines [107], it is therefore perhaps of little surprise that these bioactive molecules become sequestrated within the dentin for later release when it is demineralised during the disease process. Indeed, the components of the dentin matrix are clearly multifunctional and can stimulate multiple processes such as promoting mineralization and stimulating cell migration and activation [92, 100, 101, 108].

The extravasation and antimicrobial activity of immune cells within the pulp result in the release of molecules that, while aimed at combatting the bacterial infection, can however also cause significant collateral host tissue damage. Degradative enzymes, such as MMPs necessary for the immune cell migration through the soft tissue matrix, cause degradative damage and the increased levels of reactive oxygen species (ROS) utilized by immune cells for antimicrobial action also damage host cells and tissues. These events can contribute to the chronic cycle of inflammation as these molecules are also known to have direct proinflammatory actions. Indeed, ROS, including superoxide anions, hydrogen peroxide, and hydroxyl radicals, can stimulate cytokine release by activating the key proinflammatory intracellular signaling pathways regulated by the p38 MAPK and NF-$\kappa$B proteins in several immune and tissue structural cell types [13, 109, 110]. Notably, these pathways have become exceedingly well characterized in the proinflammatory process and are central to extracellular signal transduction in response to cellular stresses, such as infection and cytokine stimulation [111, 112]. It should however be noted that while the activation of these signaling pathways is generally regarded as being involved in the amplification of the immune and inflammatory responses, they also appear to associate with repair and regeneration signaling. Indeed, while generally it is regarded that tissue repair does not occur until infection is under control and the inflammation is modulated, the magnitude and temporospatial nature of events may be key to fine-tuning this complex response. The link between inflammation and regeneration via these intracellular signaling interactions will be further discussed below.

Notably, the dentin-pulp complex has significant regenerative potential following injury due to its tertiary dentinogenic responses. Due to the differences in complexity of the cellular processes involved in reactionary or reparative dentinogenesis, the local inflammatory response will likely have differing effects at the different stages within it [66]. It is notable that tissue reparative events will likely only occur when the infection and inflammation are under control and this may result from the immune response resolving the infection, or following clinical intervention to remove the disease. This balance between defence and repair in the tissue is clearly important. Indeed, it would not appear practical for

body resource to be utilized to rebuild tissue, which remains under attack from infection and hence may continue to break down. Furthermore, from a clinical standpoint, if the tissue is rebuilt while the infection is still present, this may prove futile and likely result in the need for retreatment.

In support of this premise, several lines of evidence indicate that chronic pulpal inflammation impedes reparative processes and the accepted paradigm is that regeneration only follows after appropriate resolution of inflammation, which likely occurs after disinfection [113–115]. Indeed, we know that while the immune-inflammatory responses aim to be protective, tissue damage occurs collaterally due to the release of degradative molecules and enzymes, as described above, and hence any reparative mechanisms ongoing may not be apparent. Potentially, the most significant evidence that resolution of infection and inflammation are necessary to enable regeneration is derived from classical animal studies, which demonstrated that repair was apparent only in artificial cavities made in germ-free animals compared with those where the cavities were infected and subsequent inflammation occurred [116]. Further evidence regarding the effects of inflammation on regeneration comes from *in vitro* studies that demonstrate the biphasic responses of pulp cells to proinflammatory signaling molecules. Notably, while relatively low levels of cytokines and growth factors can be stimulatory to cells, high levels of these molecules, such as TNF-$\alpha$ and TGF-$\beta$, present during infection and inflammation can cause cell death [97, 108, 117, 118]. More direct evidence also comes from studies that demonstrate stem cell differentiation processes are clearly impeded by proinflammatory signaling [119, 120].

Recent work has, however, indicated that inflammatory signals can stimulate repair processes (reviewed in [121]). Indeed, signal transduction via both the key proinflammatory MAPK and NF-$\kappa$B pathways (as described above) is also implicated in several reparative response processes. Data from several sources have demonstrated that these intracellular cascades can be activated in dental cells by several inflammation-related molecules, including bacterial components, ROS, and cytokines, which subsequently drive *in vitro* mineralization and differentiation responses. Arguably, it may be that acute or low levels of these inflammatory signals are necessary to signal these regenerative responses [109, 122–128]. Interestingly, it is also known that dying cells release and promote local secretion of low levels of proinflammatory mediators as damage-related signals [129]. Potentially, this sterile inflammation may occur during pulpal fibroblast senescence in the aging pulp and, subsequently, this process may generate nucleation points which drive pulp stone formation [130]. Combined, these data indicate that a delicate balance exists between the signaling or inhibition of repair and regeneration by proinflammatory mediators. Subsequently, we hypothesize that relative low level or acute inflammation may stimulate tissue regeneration, whilst higher chronic levels may impede the reparative processes and favor intense immune cell recruitment and activation.

Intriguing evidence linking the two processes of repair and regeneration can also be derived from data which demonstrates the sharing of receptors between immune and repair-related cells. Indeed, the C-X-C chemokine receptor 4 (CXCR4) is known to be expressed on both of these two different cell types [131, 132]. Furthermore, both the receptor and its ligand, stromal cell-derived factor-1 (SDF-1)/CXCL12, have been detected within the dentin-pulp complex and are reportedly upregulated during dental disease [133, 134]. Potentially, the sharing of this chemotactic receptor by these cell types appears somewhat logical as tissues which are damaged or infected, as is the case with the tooth during caries infection, need to recruit both immune and stem cells to injury sites to facilitate defence and repair [135]. The regulation as to which of these two processes predominates may, however, be locally controlled as studies have shown that cytokine levels modulate the stem cell surface expression of CXCR4. It is therefore conceivable that relatively high levels of proinflammatory molecules may abrogate CXCR4-mediated stem cell response at sites where inflammation is overriding [131].

Further support for the role of inflammation events preceding repair is potentially provided clinically following the application of the chemically related pulp capping agents of calcium hydroxide and Mineral Trioxide Aggregate (MTA). These restorative agents are known to enable the formation of tertiary dentin, in the form of a dentin bridge, beneath the site of application. Notably, however, chronologically prior to visible signs of hard tissue healing process, dental tissue inflammation is routinely observed histologically [136]. While calcium hydroxide has been applied clinically for over 60 years [137–140], its mechanism of action in the induction of reparative dentinogenesis remains controversial, although its beneficial effects have been attributed to the local release of hydroxyl ions [139], which raise pH and lead to cellular necrosis [141, 142]. Hence, it is the nonspecific chemical tissue irritation effect of these restoratives which has been cited as their principal mechanism of action for promoting dentin-pulp complex tissue regeneration. More recent studies have also indicated that these regenerative effects are perhaps more related to their ability to sterilize the site of infection whilst releasing bioactive signaling components from the dentin [143, 144]. It could therefore be hypothesized that a combination of events may occur to facilitate dentin-pulp complex repair *in vivo* following their placement. Indeed, the local cellular necrosis may stimulate sterile inflammation [145–148], which is able to resolve due to the elimination of bacteria by the combination of the material and clinical procedure. This relatively mild and acute immune response combined with the leaching of growth factors and signaling molecules from the dentin may subsequently generate a conducive environment for reparative dentinogenesis [149–152]. Furthermore, it has been observed that MTA can increase cytokine release, including IL-1$\alpha$, IL-1$\beta$, IL-2, IL-6, and IL-8, from mineralizing cells and this mild and acute material-induced inflammatory response may also contribute to clinical repair [153–155].

To better characterise the molecular response of the pulp tissue during caries, we have undertaken high-throughput transcriptional profiling using disease and healthy pulp tissue. Data indicated that the predominant tissue processes, pathways, and molecular interactive networks detected were

proinflammatory in nature, while there was minimal evidence of repair-associated molecular events [11] (Figure 3). Indeed, increased expression of many well-characterised proinflammatory mediators was detected while further data-mining enabled us to identify expression changes in several molecules previously not associated with dental tissue disease. We subsequently speculated that underlying molecular repair-related responses may be occurring and, therefore, further bioinformatically interrogated our datasets and identified the candidate repair-related molecule, adrenomedullin (ADM). This pleiotropic cytokine was upregulated during dental disease and is reported to have antibacterial and immunomodulatory properties, as well as being a known molecular mediator of angiogenic and mineralized tissue reparative processes. Others have also shown that it is able to modulate inflammation at the molecular level [156–159]. Our subsequent studies went on to demonstrate that ADM may exert similar effects within the dental tissues and is archived within the dentin during primary dentinogenesis [160]. These data indicate that this molecule may be a viable target for use in future biological therapies for both hard and soft tissue repair of the dentin-pulp complex.

While it is aimed at identifying molecular modulators of dental tissue inflammation, which may have efficacy in enabling hard tissue repair, it is also interesting to speculate that direct delivery of mesenchymal stem cells (MSCs) or their secretomes may provide a novel approach to control inflammation. Indeed, adult/postnatal MSCs, including dental pulp stem cells, isolated from a range of tissues have demonstrable immune-modulatory capability either via their cell-cell contact or via their secreted components which can inhibit proliferation, cytokine/antibody secretion, immune cell maturation, and antigen presentation by T cells, B cells, NK cells, and DCs [161–163]. Direct cell-to-cell contact between stem and immune cells is known to elicit secretion of soluble factors such as TGF-$\beta$1 and indoleamine-2,3-dioxygenase-1 which subsequently can dampen the immune response. While MSCs may provide a cell therapy approach to aid repair of inflamed dental tissue if delivered appropriately, better characterization of their secreted active components may enable identification of novel molecules for targeted dental tissue repair.

Data now indicate that, during a progressive carious infection, initially it is the odontoblasts which detect the invading bacteria and, subsequently, cells within the pulp core such as resident immune cells, fibroblasts, stem cells, and endothelial cells become involved in the molecular response. Further autocrine and paracrine signalling amplifies the reaction and leads to an increased immune cell infiltration. The elaboration of a plethora of cytokines and chemokines will have resultant consequences for the tissue and its innate repair mechanisms and this milieu is further added to by the signalling molecules released from the dentin matrix itself by the action of bacterial acids [48]. This local cocktail of bioactive molecules will continue to chronically recruit and activate immune cells, which combat the invading bacteria. The relatively high levels of proinflammatory mediators present in the local environment will likely impair any healing events at the cellular and molecular levels. Currently, the application of dental clinical procedures and restorative materials aims to remove the infection, facilitate the resolution of the inflammatory response, and enable repair processes. Notably, attempts are now being made to apply knowledge of the cytokine networks invoked for diagnostic and prognostic purposes. It is envisaged that these data will enable identification of lesions refractory to endodontic treatment due to unresolved chronic inflammation [164].

While diagnostics are being developed based on the characterisation of the inflammatory response, modulators of inflammation have the potential to be used adjunctively to facilitate the healing response and aid restoration longevity. Recent work has demonstrated that dental resin restorative procedures can be supplemented with antioxidants, such as N-acetyl-cysteine (NAC). This supplementation reportedly provides protection to the pulpal cells from ROS generated following resin placement. Interestingly, NAC may also limit the activation of the key ROS activated NF-$\kappa$B proinflammatory pathway [165] and this modulation may also minimise the inflammatory response, subsequently creating a more conducive environment for tissue repair. More studies in this area may identify other antioxidants and pathways, which may facilitate dental tissue repair responses.

Other work has demonstrated the importance of the modulation of both ROS and reactive nitrogen species (RNS) to facilitate repair. Kim et al. [166] have recently demonstrated that the anti-inflammatory mechanism of exogenously applied PPAR$\gamma$ in activated human dental pulp cells was likely due to the removal of both NO and ROS, which subsequently suppressed both the NF-$\kappa$B inflammatory and extracellular signal-regulated kinase (ERK) 1/2 signaling pathways. The anti-inflammatory effects of other naturally derived compounds, such as pachymic acid, derived from the mushroom *Formitopsis niagra,* have also been explored. Interestingly, this compound may not only have anti-inflammatory activity, but also appears to be able to promote odontoblast differentiation via activation of the HO-1 pathway. These data further indicate the important interrelationship between inflammation and repair and its potential application for dental disease treatment [167]. Recently, an exciting area relating to the therapeutic application of regulatory microRNAs (miRNAs) has been reported. These miRNA molecules have been shown to be differentially expressed between healthy and diseased dental pulps [168] and work is ongoing within the pharmaceutical industry to engineer these molecules for delivery to treat a range of inflammatory diseases. Potentially, miRNAs may therefore one day be applied in the treatment of dental disease as a means to tip the balance from a chronic inflammatory environment to one more conducive for tissue repair. It is now evident that more studies are required which target the interactions between the inflammatory and regenerative responses within the dentin-pulp complex as these may identify novel therapies for dental tissue repair.

## 4. Conclusion

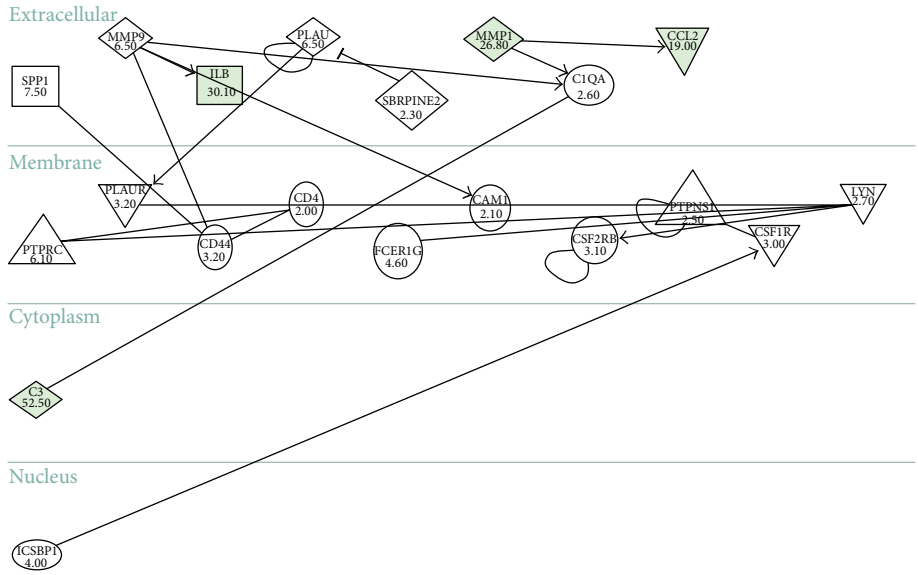
We are now developing a better and more complete understanding of the molecular and cellular events which occur

| Function | Network | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 |
| Chemotaxis* | ■ | ■ | ■ | | | | | | | | ■ | ■ | | ■ | | |
| Activation* | ■ | | | | | | | | | | | | | | | |
| Phagocytosis* | ■ | | | | ■ | | | | | | | | | | | |
| Development/differentiation* | | | | ■ | | | | | | | | | | | | |
| Respiratory burst/ROS* | | | | ■ | | ■ | | ■ | | | | | ■ | | | |
| Lymphocyte function* | | | | | ■ | | | | | | | | | | | ■ |
| Apoptosis | | | | | | ■ | | | | | | | | | ■ | |
| Calcium mobilization/flux | | | ■ | | ■ | | | ■ | | | | | | | | |
| Lipid synthesis/metabolism | | | | | | | ■ | ■ | | | | | ■ | | | |
| Hard tissue formation# | | | | | | | | | ■ | | | | | ■ | | |
| Hard tissue resorption# | | | | | | | | | | ■ | | | ■ | | | |
| NO synthesis/regulation* | | | | | | | | | | | | | ■ | | | |
| Bone marrow/cell movement* | | | | | | | | | | | ■ | | | ■ | | |

(a) Carious diseased pulp

| Function | Network | | |
|---|---|---|---|
| | 1 | 2 | 3 |
| Mitogenesis/cell cycle progression | ■ | ■ | ■ |
| Cell viability/growth | | | ■ |
| Exocytosis | | ■ | |
| Cell polarization | | ■ | |
| Angiogenesis | | | ■ |

(b) Healthy pulp




(c)

Figure 3: Tables ((a) and (b)) showing the key functions associated with the 16 and 3 molecular networks identified as being significantly activated (≥ 6 focus genes) in carious and healthy pulpal tissue, respectively. Shading of boxes indicates the networks which associated with the function and hence supported its inclusion as being active. Analysis was performed using the Ingenuity Pathways Analysis (IPA) software (http://www.ingenuity.com/products/ipa) on the high-throughput datasets reported in McLachlan et al. [11]. Sixteen and three functional categories were identified as being activated in carious diseased and healthy pulpal tissues, respectively. Carious diseased pulp tissue clearly demonstrated increased molecular network and functional activity compared with healthy pulpal tissue. Asterisks (∗) in (a) indicate functions which are associated with immune system cells (as identified by IPA); notably some evidence of hard tissue repair function was also evident (#). Ontological functions identified in (b) likely associate with pulp tissue homeostatic processes. Image (c) shows an example network (network 1 from the carious pulp tissue dataset) which also shows the subcellular localisation of the molecules that were identified as differentially expressed. The activation of this network via intracellular signalling cascades results in the elaboration of key inflammatory-associated chemokines, such as CXCL8 (IL-8) and CCL2, and the matrix metalloproteinases (MMPs) 1 and 9.

in the dentin-pulp complex during inflammation and repair following carious disease. While disinfection of the dental tissue is clearly imperative for the health of the tooth, the subsequent interaction between dental tissue defence and repair is complex and the fine-tuning of the regulation of these processes is important for ensuring which response predominates when vital pulp tissue can be clinically retained or regenerated. It is clear that sustained research activity in this area combined with clinical translational approaches may result in the development of new therapeutics which enable host defence and repair events. Advances in our understanding of the interactions between immune and regenerative responses may therefore influence clinical practice and benefit dental patients in the future.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

## Authors' Contribution

Jean-Christophe Farges, Brigitte Alliot-Licht, and Paul R. Cooper contributed equally to this work and should be considered co-first authors.

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