**SIGNIFICANCE**

To improve the efficacy of regenerative endodontic procedures (REPs), high-quality basic science should underpin developments in clinical treatment. A molecular understanding of the interaction between dental infection, inflammation, and repair is essential if we are to create novel treatments. The use of locally applied pharmacologic inhibitors targeted at NETs, the inflammasome and epigenetic processes, and phototherapeutic strategies, present exciting opportunities to develop next-generation solutions, which will improve the outcome of vital pulp treatment and REPs.

**ABSTRACT**

**Introduction:** The improvement of regenerative endodontic procedures requires an understanding of the key clinical questions combined with a fundamental biological knowledge of how the dental tissues behave during health, disease, and repair. Therefore, partnerships between clinicians and basic scientists are essential to drive the field forward and improve patient outcomes. **Methods:** This review aimed to provide a background to dentin-pulp biology and the interaction between infection, inflammation, and regeneration. **Results:** We have highlighted how the release of neutrophil extracellular traps (NETs) within the pulp are double-edged; while they aim to limit the bacterial infection, they may actually exacerbate cell death and chronic inflammation. Aberrant levels of these structures may occur because of ineffective host immunologic processes, viral infections, or impaired clearance caused by bacterial virulence factors. We also postulate a proinflammatory link in the pulp between NETs and the inflammasome activated by pathogen-associated molecular patterns and damage-associated molecular patterns. Subsequently, we discuss areas potentially fruitful for future clinical exploitation involving NET inhibitors, inflammasome modulators, phototherapies, and novel epigenetic approaches. **Conclusions:** Sustained scientist-clinician research partnerships along with an increased understanding of the association between inflammation and regeneration within the dentin-pulp complex will lead to future patient benefit. *(J Endod 2020;46:S10–S18.)*

**KEY WORDS**

Mesenchymal stem cells; pulpitis; regenerative endodontics; tissue engineering; vital pulp treatment

**OVERVIEW**

**Dental Disease: Infection, Inflammation, and Tissue Repair**

Dental caries affects >90% of the adult global population, and its progression leads to pulpitis. Even during the early stages of microbial infection, bacterial components can diffuse through dentinal tubules and induce localized pulp inflammatory responses. As the carious infection progresses, the immune response intensifies and the bacterial microflora composition adapts, becoming predominantly anaerobic within deeper lesions. Ultimately, if unchecked, the infection will potentially lead to irreversible pulpitis, pulp necrosis, and ultimately apical periodontitis.

A complex molecular reaction in response to infection occurs within the pulp. The cells and tissues within it express receptors that recognize components of the infecting agents, with the host response aimed at bacterial elimination. Odontoblasts as well as resident pulp cells, including fibroblasts, neurones, immune, endothelial, and stem cells (SCs), express a range of receptors able to detect bacterial components, such as lipopolysaccharides (LPSs), lipoteichoic acids (LTAs), and bacterial DNA. The best characterized family of receptors are those of the Toll-like receptor (TLR) family. Ten human TLR members have been identified that detect both microbial components and host-derived damage signals, and once the ligand is bound, key transcriptional regulatory pathways are initiated, such as those involving nuclear factor kappa B and mitogen-activated protein kinase. Activation of these pathways leads to the release of antimicrobial molecules and immune regulator cytokines. We and others have shown that the diseased pulp expresses elevated levels of cytokines, which regulate immune cell recruitment, extravasation, activation, differentiation, and antibody production. Notably, key cytokines involved in pulp inflammation include interleukin (IL)-1α, IL-1β, IL-4, IL-6, IL-8, IL-10, transforming growth factor beta 1 (TGF-β1), and tumor necrosis factor alpha (TNF-α). The chemokines/cytokines released...
locally, along with vasoactive molecules, generate signals and gradients that direct immune cell populations, such as neutrophils, to traffic from the bloodstream to the infection site. Neutrophils localize to the tissue provide a first line of defense and mediate bacterial killing using a variety of mechanisms which are described later. Recruited macrophages will also remove bacteria and cellular debris (efferocytosis) and can drive resolution of inflammation via their M1 to M2 phenotypic conversion. Subsequently, M2 phenotype cells release anti-inflammatory cytokines, such as TGF-β and IL-10, which subsequently enable regenerative wound healing processes3.

It is widely accepted that inflammation is double-edged because it restores tissue homeostasis after infection while also causing collateral tissue damage and impeding the repair process while it ensues. Indeed, this link between inflammation and regeneration is becoming increasingly recognized as highlighted by the growing number of publications (Fig. 1A and B). The widely accepted paradigm both in the pulp and at other bodily sites is that healing can only occur after removal of the infection, enabling a significant dampening of inflammation10–12. Furthermore, animal and in vitro studies have shown the biphasic effects of inflammatory mediators13. At relatively low levels, cytokines such as TNF-α and TGF-β1 as well as reactive oxygen species (ROS) and bacterial components can stimulate dental cell-mediated repair mechanisms. However, when these same molecules are present at higher levels, such as during chronic inflammation, they exert deleterious effects, such as the induction of pulp cell and tissue death. Furthermore, stem cell-mediated repair processes can be directly impeded by proinflammatory molecules14.

Notably, without clinical intervention, it is likely that the pulp will lose vitality because of the infection and associated chronic inflammation. Pulp necrosis may occur, which will require intervention such as root canal treatment (RCT). RCTs are technically difficult, potentially problematic, can be relatively expensive, and may weaken tooth structure15. Although RCT is a relatively common dental procedure, it is not always effective in the long-term, with relatively high failure rates of up to 30%–50%, particularly if performed by general dental practitioners15. Risk factors for treatment failure include inefficient chemomechanical debridement as well as certain individuals exhibiting compromised innate repair responses, as is seen in diabetic patients16–18.

The two forms of host-driven dental repair are well described, with mild tissue injury, such as an early carious lesion, resulting in the up-regulation of the original surviving odontoblast secretory activity in a process termed reactionary dentinogenesis. The newly formed dentin is tubular and continuous with the original primary and secondary dentin layers. However, with a greater intensity of injury, such as during a more rapidly progressing carious lesion, the primary odontoblasts undergo cell death beneath the lesion and are replaced by odontoblast-like cells derived from SCs from within, or migrating into, the tooth. Clearly, the disease must be arrested or modified (eg, selective/noneselective caries removal) to enable these processes. The recruited and newly differentiated cells deposit a tertiary and more disorganized dentin matrix, clinically seen as a mineralized bridge beneath a suitable capping restoration (eg, calcium hydroxide or hydraulic calcium silicate) in a process termed reparative dentinogenesis. Both tertiary dentinogenic processes wall off the invading infection, preserving pulp vitality and restoring the tooth’s structural integrity and functionality19–21.

It is established that dental tissue repair processes can be signaled by bioactive molecules released from the dentin by caries-derived bacterial acids or restorative agents, such as calcium hydroxide and calcium silicates. Because of the biphasic signaling that occurs in response to molecules, such as TNF-α and TGF-β1, along with ROS and bacterial components, it is apparent that a fine balance exists between pathogenesis and dental tissue repair16–20.

**Neutrophils, NETosis, and Dental Disease**

Previously neutrophils are well described in terms of their release of antibacterial molecules, such as ROS, as well as in their degranulation of antimicrobial peptides (AMPs). They also express tissue degradative enzymes, such as matrix metalloproteinases, to enable cell movement through the tissue. Although the release of these molecules ultimately aims to restore tissue homeostasis, dysregulated levels cause collateral damage to tissues, cells, DNA, and proteins17. More recently, a relatively novel antibacterial killing and containment mechanism termed *neutrophil extracellular traps* (NETs), has been described29. This process involves ROS-triggered release of cellular DNA adorned with AMPs. Recent evidence indicates that if this process is dysregulated, chronic inflammation can ensue, which leads to further cell and tissue damage. Our studies have now shown that NETs are present within the diseased dental pulp, and their release may cause cellular insult, leading to inflammation exacerbation as well as initiating pulp cell death30,31.

NETs comprise web-like structures of decondensed nuclear chromatin DNA adorned with antimicrobial molecules, including histones and granule-derived AMPs, proteases, and enzymes responsible for ROS generation29,32–35. Notably, ROS generation underpins the early-stage signaling necessary for NET production36,37, and with the subsequent unpacking of the nuclear chromatin achieved by the enzymatic action of the calcium-dependent enzyme peptidylarginine deiminase 438. This enzyme catalyzes a cullurnification process transforming positively charged arginine residues in the packing histones into neutrally charged citrulline residues. The loss of the electrostatic attraction between the DNA and the histones subsequently facilitates the nuclear material to be actively released extracellularly in the form of NETs39.

Subsequently, it is the electrostatic charge interactions between the released NET-DNA and the bacterial outer membrane/ wall that result in entrapment. This attraction enables the colocalization of high concentrations of antimicrobial molecules, which facilitates the bacteriocidal effect50. The activation of multiple signaling receptors is required for NET release, including stimuli of bacterial components, nitric oxide, cytokines/chemokines, yeasts, fungi, protozoa, AMPs, antibodies, and complement, indicating that the process is tightly regulated and represents a “last resort” killing mechanism11. Because of its similarities with other programmed cell death processes, such as apoptosis and necrosis, the process has subsequently been termed ‘NETosis’39. Interestingly, in 2009, it was shown that the expulsion of mitochondrial NETs (as opposed to nuclear DNA derived NETs) enabled cells to remain viable and required less potent stimulus while providing a rapid antimicrobial strategy42.
LTA modification on Gram-positive bacteria consequently enables decreased interactions with NETs. Notably, some bacteria appear to stimulate relatively low levels of NETs, and, interestingly, our analyses have shown that several dental disease-relevant strains may also use this simple strategy to decrease NET induction\(^3\). Clearly, within the dental pulp, it appears that different bacteria may use a variety of approaches to evade NETs. Deoxyribonuclease expression by specific bacteria at different stages of growth may confer benefits to the whole microbial community, and this may enable further propagation and dissemination of bacteria within endodontic tissues\(^4\). Furthermore, certain bacteria, such as *Enterococcus faecalis*, *Porphyromonas gingivalis*, and *Prevotella intermedia*, have been shown to exhibit surface modifications including the presence of a polysaccharide capsule, and this phenotype strongly associates with persistent root canal biofilm infections\(^5\)–\(^8\).

**NET Effects in Host Tissues**

Although NETs are aimed at protecting the host from invading bacteria, their presence has been associated with several autoimmune and autoinflammatory pathologies. Excess and aberrant NET release or defective clearance provides a source of proinflammatory and cytotoxic molecules, whereas in the autoimmune disease, systemic lupus erythematosus, neutrophil-derived granular proteins associated with NETs, including myeloperoxidase and proteinase 3, can cause autoantibody responses that limit NET clearance\(^9\). Furthermore, inefficient removal of NETs may provide a reservoir of peptidylarginine deiminase 4 hypercitrullinated proteins, which result in antigens contributing to chronic inflammatory disease\(^10\)–\(^12\). Interestingly, *P. gingivalis*, which is frequently found in endodontic infections, possesses its own peptidylarginine deiminase enzyme\(^13\), which may citrullinate its own or host proteins, potentially contributing to the auto-inflammatory cycle within root canal systems.

Recent animal studies of lung disease have shown that NETs and their components cause significant local tissue and endothelial cell damage. This effect was particularly evident in animals that had both an influenza virus and *Streptococcus pneumoniae* dual infection and were associated with a compromised macrophage clearance of NETs\(^14,15\). Further analyses indicated that the viral infection led to neutrophil priming, which resulted in hyperactive NET release. Dual-infected animals subsequently exhibited decreased survival because of increased lung tissue damage, which was associated with exaggerated inflammation compared with the single viral or bacterial infected animals\(^16,17\). Although several immunogenic molecules described previously could contribute to this pathogenicity, the role of histones is recognized in this process\(^18\). We and others\(^19\) have shown that histones not only have antibacterial activity, including killing of endodontic infection-associated bacteria, but also they act as damage-associated molecular patterns (DAMPs) when released extracellularly.

There are 5 histone types responsible for the packaging of nuclear DNA into chromatin, and they are categorized into 2 groups: core histones (H2A, H2B, H3, and H4) and linker histones (H1 and H5)\(^20\). NETosis along with apoptosis and necrosis have been shown to result in their release, and local cells detect their presence extracellularly by their binding to receptors, such as TLR-2, -4, and -9 as well as via the inflammasome. Subsequently, this binding triggers activation of multiple signaling pathways (eg, nuclear factor kappa B, mitogen-activated protein kinase, NLRP3, and caspase-1) in a single or combined manner and invokes several processes such as proinflammatory signaling and cell death induction. Notably, high concentrations of histones have been detected...
in several inflammatory and infectious diseases, and inadequate clearance of this cell debris by macrophages may contribute to pathogenicity. Consequently, extracellular histones are considered mediators of several inflammatory diseases and are being further investigated as biomarkers for disease progression and as therapeutic targets.31

Recently we have shown that histones, such as H2A or combinations of histones (but not DNA alone), are cytotoxic to human pulp cells and can drive cytokine release, which aims to recruit macrophages via CD14 signaling to remove cell debris.31 However, potentially in the presence of endodontic bacterial virulence factors, such as gingipains derived from P. gingivalis, macrophage homing is impaired, and cell debris clearance becomes inefficient due to the cleavage of CD14 from the cell surface.37 The subsequent frustrated efferocytosis may result in failure of the macrophage M1 to M2 repair-oriented conversion, thereby perpetuating local tissue chronic inflammation (Fig. 2A and B). As described previously, it is notable that dual infections of bacteria with viruses may lead to elevated NET levels, leading to a more rapid destruction of the affected tissues. The report that viral RNAs have been detected in diseased pulp adds further credence to the possibility that NETs may be important in chronic pulpal inflammation.

Future Endodontic Therapies

The pulp has an innate regenerative and healing capability if a conducive environment is provided, and, therefore, minimally invasive biologically-based dental procedures are being developed.38 Vital pulp treatments are a component of regenerative endodontic procedures, which aim to maintain and support the pulp tissue that would previously clinically have been removed. Traditionally, VPT has been associated with poor outcomes, potentially because of

1. a lack of understanding of pulp biology,
2. functionally inadequate dental restorative materials, and
3. the absence of suitable diagnostics that can indicate the diseased state of the pulp.39

Along with the development of novel material approaches, SC and growth factor-based therapies are being developed. The understanding of the impact of inflammation on the repair processes is also essential for new treatment development, and the potential of several of these areas are discussed later.

NET Modulators

Studies by ourselves and others have focused on identifying NET inhibitors as the dysregulated release, and inefficient clearance of these structures may perpetuate chronic inflammation and compromise tissue healing, as described previously.31 In a recent study, we reported the development of a high-content biology approach to screen a pharmaceutical compound library and subsequently identified several chemical modifiers of NET release that induced, inhibited, or exerted biphasic effects dependent on dosage. Although these off-label drugs could be used in a range of diseases, there is the potential that they may also have efficacy in the treatment of pulpal disease as an adjunct to conventional therapies by modulating inflammation. Of the compounds that were shown to exert an inhibitory effect on NETosis, including laptapin, carmustin, erlotinib, bosutinib, rapamycin, and nilotinib, to our knowledge, only rapamycin has been studied in dentin-pulp biology. Indeed, data from one recent study indicated that this compound was able to enhance the dentinogenic capacity of stem cells in culture.64 Clearly, further studies on the relevance of NETs and their components in pulpal inflammation are still required along with assay of the effects of novel therapeutics, which may modulate inflammatory processes and enhance dental tissue repair. In addition, the contribution of NET components, such as histones, to chronic pulpal inflammation should be characterized along with their utility in determining the diseased state of the pulp.

Inflammiasome Inhibitors

An expanding area of cutting-edge research relates to understanding the role of the inflammasome in a range of immune and inflammatory diseases.65 The expression of master regulatory cytokines IL-1β and IL-18 requires 2 signals generated via inflammasome signaling (Fig. 3). Notably, along with stimulation from a pathogen-associated molecular pattern (PAMP), a second signal derived from a DAMP is also required. A variety of PAMPs, such as LPS, bacterial DNA, or LTA, can provide the first signal, whereas the second signal is derived from cell death-associated molecules, such as adenosine triphosphate or extracellular histones along with other NET components, such as neutrophil elastase and proteinase 3. Intracellular signaling is subsequently activated by both arms of the pathway resulting in pro-cytokine generation; subsequent post-translational processing by caspase 1 is required to enable their extracellular release.

Currently, there are only limited studies on the role of the inflammasome in the dental pulp; however, recent work has shown that irreversible pulps exhibits increased NLRP3/caspase 1 activity compared with both healthy pulp and reversible pulps.66 These findings are consistent with previous data showing chronic and elevated IL-1β production in diseased pulp. Intriguingly, the overlap between NET release and clearance with inflammasome activation (Fig. 3) identifies a potential link as to how dysregulation of these processes could lead to persistent pulpal disease.65

Studies are now underway to identify inhibitors of inflammasome signaling, and although some compounds can be targeted more specifically, such as for NLRP3, other strategies are relatively broad spectrum, such as pan-caspase inhibitors.67 More studies on the role of the inflammasome in pulpal disease are warranted along with a determination of whether modulation of this signaling will facilitate innate regenerative mechanisms.

Phototherapy

The application of low-level light therapy or photobiomodulation therapy to limit inflammation and promote tissue repair has provided considerable focus. Although this technology is more widely applied in the treatment of other diseases, there is significant potential for its application in dentistry to not only modulate inflammation and pain but also to promote tissue repair processes. Recent in vitro and in vivo studies using a range of wavelengths (within the 400- to 1100-nm spectrum) delivered at low doses using lasers or light-emitting diodes have shown both anti-inflammatory and tissue regenerative actions in the dentin-pulp complex.68 There is also significant potential for the direct use of phototherapy to control infection on the tooth surface and within it. In particular, wavelengths of 405 nm have shown promise in terms of having direct antibacterial potential because of their effect on bacterial endogenous porphyrins, which when activated release ROS.69 In addition, photodynamic therapies also show potential for managing endodontic infections.70

However, photodynamic therapy acts indirectly as the irradiation is used to activate a photosensitizer, which locally releases damaging ROS molecules. Phototherapies offer significant potential in endodontics, and sustained research combining expertise of biological scientists, experts in photophysics, and clinicians is required.71

Epigenetics

Epigenetic modifications cause transcriptional changes which are not due to alterations in DNA sequence, and DNA methylation and
Histone modifications (methylation and acetylation) are the most frequently studied. Other epigenetic influences on gene expression include noncoding RNAs (eg, microRNA and long noncoding RNAs). Epigenetic mechanisms not only control SC and mineralized tissue repair processes but also play a role in the regulation of inflammation. DNA methylation involves the transfer of a methyl group to a cytosine base of DNA to generate 5-methylcytosine. In humans, four DNA methyltransferase enzymes (DNMTs) regulate this process. For histone acetylation, acetyltransferases add a negatively charged acetyl group to weaken the interaction between DNA and its packaging histones, a process balanced by histone deacetylase (HDAC) activity, which removes the acetyl group. Both these processes regulate DNA accessibility and thereby modulate gene expression.

Only one study so far has investigated how DNMT inhibitors (DNMTis) affect reparative dentinogenesis, with data indicating that therapeutic application promoted the induction of an odontoblast-like cell phenotype in dental pulp cell cultures. Notably, clinically-based studies have linked changes in DNA methylation patterns with the inflammatory process in the dental pulp. Complementary in vitro work has also shown the potential role of DNMTs in bacterial-induced inflammation in dental pulp cells. Several HDACis have already shown efficacy in DPCs in vitro in promoting SC responses. In addition, an in vivo study has also reported that HDACi application promotes dentin deposition.

Consequently, HDACis and DNMTis are postulated to provide an opportunity to develop topically placed, inexpensive bioinductive epigenetic-based therapies for dentin-pulp complex repair.

**CONCLUSION**

Current dental treatments for the damaged and inflamed pulp are limited because of partial understanding of the processes that occur during disease and repair. Sustained and ongoing scientist-clinician research activity into the understanding of these biological processes is required to enable exploitation of new and robust restorative therapies that benefit patients.
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