Inflammation and regeneration in the dentin-pulp complex: net gain or net loss?

Cooper, Paul; Chicca, Ilaria; Milward, Michael

DOI:
10.1016/j.joen.2017.06.011

INFLAMMATION AND REGENERATION IN THE DENTIN-PULP COMPLEX: NET GAIN OR NET LOSS?

PR Cooper*, IJ Chicca, MJ Holder & MR Milward

Oral Biology, School of Dentistry, College of Medical and Dental Sciences, 5 Mill Pool Way,

Edgbaston, Birmingham B5 7EG, UK

Key words: Pulp, dentine, neutrophil extracellular traps, polymorphonuclear leukocytes, reactive oxygen species, granulocytes

Corresponding author:

*Paul R. Cooper

Oral Biology,

School of Dentistry,

The University of Birmingham,

5 Mill Pool Way,

Edgbaston,

Birmingham,

UK

B5 7EG

Direct Dial: +44 (0) 121 466 5526

Secretary: +44 (0) 121 466 5073

ABSTRACT

The balance between the immune/inflammatory and regenerative responses in the diseased pulp is central to clinical outcome and this response is unique within the body due to its tissue site. Cariogenic bacteria invade the dentin and pulp tissues triggering molecular and cellular events dependent on the disease stage. At the early onset, odontoblasts respond to bacterial components in an attempt to protect the tooth's hard and soft tissues and limit disease progression. However as disease advances the odontoblasts die and cells central to the pulp core, including resident immune cells, pulpal fibroblasts, endothelial cells and stem cells, respond to the bacterial challenge via their expression of a range of pattern recognition receptors which identify pathogen associated molecular patterns. Subsequently there is recruitment and activation of a range of immune cell types, including neutrophils, macrophages, T- and B-cells, which are attracted to the diseased site by cytokine/chemokine chemotactic gradients initially generated by resident pulpal cells. While these cells aim to disinfect the tooth, their extravasation, migration and antibacterial activity [e.g. release of reactive oxygen species (ROS)] along with the bacterial toxins cause pulp damage and impede tissue regeneration processes. Recently, a novel bacterial killing mechanism termed Neutrophil Extracellular Traps (NETs) has been described which utilizes ROS signaling and results in cellular DNA extrusion. The NETs are decorated with antimicrobial peptides (AMPs) and their interaction with bacteria results in microbial entrapment and death. Recent data demonstrate that NETs can be stimulated by bacteria associated with endodontic infections and they may be present in inflamed pulp tissue. Interestingly some bacteria associated with pulpal infections express DNase enzymes which may enable their evasion of NETs. Furthermore, while NETs aim to localize and kill invading bacteria using AMPs and histones, limiting the spread of the infection, data also indicate that NETs can exacerbate inflammation and that their components are cytotoxic. This review considers the potential role of NETs within pulpal infections and how these structures may influence the pulp's vitality and regenerative responses.

INTRODUCTION

Previously we have described how the pulp's response to infection and injury is similar to that of many other tissues in the body [1]. Cells of the dentin-pulp complex detect invading bacteria by their expression of a range of pattern recognition receptors (PRRs), which identify pathogen associated molecular patterns (PAMPs). The PRRs reported as being present in the pulp include Toll-like receptors (TLRs), Nucleotide-binding oligomerization domain (NOD) -1 and -2 proteins and the Nod-like receptor (NLR) family member pyrin domain containing 3 (NLRP3) complex, also known as the inflammasome. The expression of many of these molecules has been shown on odontoblasts, pulp fibroblasts, pulp stem cells, neurones and endothelial cells, and they are able to detect several components of the invading bacteria ranging from their DNA to outer membrane components, such as lipopolysaccharides (LPSs) [2-12]. Once host cells have detected bacterial components, they induce the expression of antimicrobial peptides (AMPs) as well as invoking the inflammatory cascade with both processes aimed at containing, and ultimately eradicating, the infection [13-15]. Initially, due to their location at the periphery of the pulp, it is the odontoblasts [16] that are the first responders; however, as the infection advances, cells deeper in the pulp core, including pulp fibroblasts, endothelial cells and stem cells, also become involved in the defense reaction [4,5]. In addition there are immune cells resident in healthy pulp tissue, such as dendritic cells and mast cells, that act as sentinels and also orchestrate the early local immune response [17-21].

At the molecular level, detection of bacterial components via the PRRs results in the activation of intracellular signaling cascades, with the primary effects being mediated via the NF-κB and p38-MAP-kinase proteins [6,7,22,23]. These pathways ultimately culminate in the translocation of master regulatory transcription factors, such as AP-1, STAT1 and NF-κB, from the cytoplasm to the nucleus where they activate the gene expression of pro-inflammatory cytokines and chemokines such as interleukin-1α, -1β (IL1-α, -β), tumor necrosis factor-α (TNF-α), IL-4, IL-6, IL-8 and IL-10. Notably, this pool of inflammatory mediators can be added to by the cytokines released by bacterial acids during the carious disease process [13-15,24,25]. Subsequently, these molecules induce both autocrine and paracrine effects which amplify the inflammatory and immune responses, and in particular generate chemotactic gradients which lead to the recruitment of immune cells from the vasculature, including T- and B-lymphocytes, plasma cells, neutrophils, monocytes and macrophages [17-19]. Notably, the extravasation, migration and antimicrobial defense responses of these immune cells can lead to significant collateral tissue damage. This response combined with the increasing infection, significantly affects the vitality of the pulp tissue and results in extracellular matrix (ECM) breakdown and death of resident cells.

During early stages of disease or when the infection has been minimized, either by the host's immune response or by clinical intervention, the tooth tissue may evoke tertiary dentinogenic responses. As has been described in detail elsewhere [26], these responses can be relatively simple in the form of reactionary dentinogenesis, which involves the direct activation of the existing primary odontoblasts, or the response may be relatively more complex in the case of reparative dentinogenesis, which involves orchestrated stem cell responses culminating in the generation of new odontoblast-like cells. We have also previously reviewed and described the links between the inflammatory and the tertiary dentinogenic responses as there is clear crosstalk between the two processes [26-28]. Indeed, it appears evident that many molecules that signal the inflammatory response, such as bacterial components, cytokines, complement and reactive oxygen species (ROS), can also stimulate aspects of tertiary dentinogenic responses [29-35]. Furthermore, signaling pathways, such as the p38-MAP-kinase cascade, are also activated during both processes [36]. Subsequently it appears likely that the activity of the intracellular signaling cascades and associated cell responses are dose and context specific. Potentially, the relatively low doses of stimuli, present during the early or resolving stages of disease stimulate regenerative responses, while more intense stimuli, which occur during active and chronic disease, inhibit regeneration. Interestingly, during incipient disease, when inflammatory levels are likely relatively low, repair responses elicited may also serve in generating a physical barrier of dental hard tissue which "walls off" the invading bacteria. The dosage effects and responses discussed above would appear to be somewhat intuitive as it would not be appropriate and potentially result in a waste of cellular resource to attempt to rebuild the damaged tissue while the infection and immune responses are both raging. Notably, the clotting and haemostatic responses will also ensue within the dentin-pulp complex to limit blood loss and provide a scaffold for later tissue repair. Interestingly, knowledge of this process is being exploited for the development of new scaffold materials that provide a framework to stimulate stem cell-based repair responses [37,38].

Innate immune response to dental tissue infection

Up to 700 bacterial species have been reported in the oral cavity with individuals harboring up to 200 different species per individual [39]. High-throughput nucleic acid sequencing approaches have shown that endodontic infections are highly complex and diverse and can contain well over 100 bacterial genera from several different phyla [40-43]. Their polymicrobial nature is dominated by Gram negative obligate anaerobic bacteria which form complex biofilms extending into dentinal tubules and the root canal network. Notably, likely due to environmental similarities, e.g. anaerobic and nutrient availability, many of the bacteria present in deep endodontic infections are also present in periodontal infections [39]. The composition and distribution of this biofilm within the tooth's root system make it clinically challenging to eliminate all invading microorganisms [44]. As described above, the dental tissue mounts

its own innate immune response which aims to eradicate the infection and restore inflammatory levels to those conducive for tissue repair (***Figure 1***). Similar to wound infection occurring at other sites in the human body, it is neutrophils [polymorphonuclear leukocytes (PMNs)] that are abundantly recruited and provide the first line of defense in the innate immune response in the pulpal tissue [21,45,46]. Neutrophils initially mature in the bone marrow, and it is estimated that even during health ~1-2 $x10^{11}$ cells are generated per day [47]. Due to their role and the increased demand placed upon the immune system during infection, their levels released into the bloodstream increase, the cells also become primed and their longevity increases [48]. When circulating and surveying for microorganisms, neutrophils reportedly have an average lifespan of ~5.4 days, following which point they subsequently undergo apoptosis and are removed by macrophages [49]. Their priming, prior to reaching the site of infection, is important as it aids their rapid response for pathogen clearance. This peripheral priming is achieved by activation by various cytokines, growth factors, complement or bacterial components. As described above, during infection a chemotactic gradient within the diseased pulp is generated by cytokines, such as IL-8, complement components and bacterial peptides (f-Met-Leu-Phe), which instruct the neutrophil to leave the circulation and traverse to the site of infection. The process of neutrophil recruitment involves the steps of tethering, rolling, adhesion, crawling and, finally, transmigration. The process is initiated by changes on the surface of the vascular endothelium and this is mediated by pro-inflammatory mediators released from tissue resident cells or by PAMPs. Notably, while neutrophils aim to combat the invading bacteria, it is known that they can also be one of the most significant mediators of local host tissue damage due to their release of ROS and proteolytic enzymes as they traverse the tissue to the site of infection [50].

Neutrophil antibacterial mechanisms

Once at the site of infection, neutrophils can utilize an antimicrobial armamentarium that exploits both intra- and extracellular killing mechanisms (***Figure 2***) and they have at their disposal significant antimicrobial proteins and molecules. Following contact with bacteria, the neutrophil can undertake phagocytosis and encapsulation into phagosomes. The neutrophil then destroys the pathogens by intracellular release of ROS (via NADPH oxygenase-dependent mechanisms) or AMPs, such as cathepsins, defensins, lactoferrin and lysozyme. Notably, these AMPs are not only released by the neutrophil granules into phagosomes but also into the extracellular milieu. Hence, degranulation can provide an extracellular killing mechanism however it may also cause further host collateral tissue damage [51-53].

Human neutrophils consecutively form three types of granules, packed with pro-AMPs and inflammatory proteins, during their cellular maturation. Azurophilic (or primary) granules, contain myeloperoxidase (MPO and azurocidin), specific (secondary) granules, contain lactoferrin, and

gelatinase (tertiary) granules, contain matrix metalloproteinase 9 (MMP9; also known as gelatinase B). Notably, azurophilic granules can be further subdivided depending on peroxidase and defensin content. Specific granules can also be further subdivided based on lactoferrin, cysteine-rich secretory protein 3 (CRISP3), gelatinase and ficolin content. Multiple types of neutrophil granules are generated as many of the proteins described cannot exist together in the innate form due to proteolytic interactions [54].

Neutrophil Extracellular Traps

NET Biology

In 2004, a novel extracellular mode of neutrophil-mediated pathogen containment and killing was described which was termed neutrophil extracellular traps (NETs) [55]. NETs are web-like structures containing de-condensed nuclear chromatin adorned with antimicrobial molecules, including histones and the AMPs derived from azurophilic specific and gelatinase granules [55]. Proteins demonstrated as being associated with NETs include i) AMPs, such as lactoferrin, cathepsin G, defensins, LL37 and bacterial permeability increasing protein, ii) proteases, such as neutrophil elastase, proteinase 3 (PR3) and gelatinase, and iii) enzymes responsible for ROS generation, such as myeloperoxidase (MPO) [56-59]. The electrostatic charge interactions between the core DNA and bacterial outer membrane/wall is understood to enable the interaction with bacteria. This 'stickiness' extends over areas of several microns due to the structure of the NETs and enables entrapment of bacteria moving within the tissue microenvironment and subsequently facilitates the co-localisation of high concentrations of antimicrobial molecules [60].

NETs are only released by mature neutrophils and their formation is impaired in neonates, which may predispose them to infection [61]. It is also evident that multiple signaling receptors (e.g. for bacterial components, cytokines/chemokines and complement) need to be triggered for NET release (see below). This indicates the need to tightly regulate this process as it likely represents a 'last resort' in neutrophil killing. Indeed, this process represents a form of programmed cell death which is termed NETosis and is distinct from apoptosis and necrosis [62]. Notably, however in 2009, Yousefi and colleagues demonstrated that the expulsion of mitochondrial NETs (as opposed to nuclear DNA-NETs) provides a means for cells to remain viable. The mitochondrial NET release process arguably requires less potent stimulation, potentially providing a relatively rapid anti-microbial strategy, which complements other neutrophil killing mechanisms [63].

Signaling of NET release

ROS release represents an important antimicrobial strategy deployed by neutrophils. Interestingly, the generation of ROS underpins the signaling for NET production, indicating the association between these two antimicrobial strategies. As described in detail elsewhere [64], the ROS signaling pathway which utilizes nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase assembly, superoxide and hydrogen peroxide production, and subsequent conversion to hypochlorous acid (HOCl) by MPO, is necessary for NET release [65]. The importance of ROS signaling in regulating NET release is further highlighted in chronic granulomatous disease (CGD). Previously, only patients' lack of ROS production was thought to be responsible for susceptibility to infection; however, more recently, their impaired NET production has also been shown implicated in their impaired infection control [66].

The next relatively well characterised step in the regulation of NET production is the de-condensing of the nuclear chromatin which is achieved by enzymatic action. Knock-out mice for the calcium-dependent enzyme, Peptidyl arginine deiminase-4 (PAD4), cannot make NETs under normal physiological conditions indicating the essential activity of this enzyme [67]. This citrullination process transforms positively charged arginine residues in histones to neutrally charged citrulline, leading to the loss of electrostatic attraction between the DNA and its packaging proteins [68]. Additionally, another mechanism for NET formation has also been described whereby primary granule-derived neutrophil elastase enters the nucleus and partially degrades histones enabling subsequent binding of MPO derived from the same granules, resulting in decondensation of the chromatin [69]. These series of events are also proposed to be triggered by ROS generation and both processes lead to chromatin de-condensation proceeding to nuclear morphological changes, nuclear membrane breakdown and associate with the neutrophil granules releasing their cargos. Subsequently, the DNA and antimicrobial proteins and enzymes combine with the chromatin in the cytoplasm prior to the rupturing of the neutrophil outer cell membrane and expulsion of these constructs [56]. Notably, the demonstration of MPO and/or neutrophil elastase co-located with DNA is important in identifying the presence of NET structures within tissues [55].

NET Stimuli

The stimuli for NETs are complex and varied. Furthermore, the temporality and context of NET induction and release is crucial as aberrant release can impede other immune functions, such as chemotaxis and phagocytosis, as well as leading to downstream pathogenic events. Currently a range of disease relevant stimuli for NET production have been reported and include nitric oxide, cytokines, bacteria and their components, such as LPS and bacterial toxins, yeasts, fungi, protozoa, AMPs, antibodies, activated platelets and statins (reviewed in detail in [64]). Whilst not necessarily having

physiological relevance, many *in vitro* studies utilize phorbol 12-myristate 13-acetate (PMA) as a stimulus for NETs (***Figure 3***). PMA works efficiently and directly, by bypassing receptor–ligand binding on the neutrophil surface, and activates cytoplasmic protein kinase C signaling which leads to intracellular ROS generation, which stimulates approximately one third of neutrophils to release NETs by 4-hours [60,62].

Gram-positive and Gram-negative bacteria stimulate NET release [55] and their components, such as LPS, can either directly induce NETs or can indirectly cause NET release via platelet activation [70]. Data from our group has shown that several bacteria and their components associated with endodontic infections are able to directly stimulate significant NET release *in vitro* albeit at levels considerably lower than those observed following PMA stimulation (***Figure 3***; & [71]). Many of the host-derived pro-inflammatory mediators previously described as playing a role in pulpal inflammation, including TNFα, IL-1β and IL-8, have also been reported to induce NET formation [72]. Furthermore, the AMP, LL-37/cathelicidin, previously reported as being involved in pulpal disease, can directly induce NET production as well as increasing NET release in response to bacteria [59].

Microbial evasion of NETs

The ionic interactions between the bacteria and NET-DNA is understood to cause microbes to become ensnared and preliminary data from our group have shown that bacteria present in endodontic infections are also susceptible to this entrapment (***Figure 3;*** & [73]). However, as would be predicted by the host–pathogen co-evolutionary arms race, bacteria have subsequently developed virulence traits, which facilitate NET evasion. DNase enzymes expressed by bacteria have now been shown to confer resistance to this antimicrobial strategy. Indeed, studies in mice using *Streptococcus pneumoniae* demonstrated that the strain expressing the wild-type EndA DNase exhibited 20% increased resistance compared with the EndA deletion mutant. The wild-type strain also demonstrated an increased spread of infection into the lungs and bloodstream compared with the EndA deletion mutant strain [74]. Others have examined the role of DNase expression in NET evasion in group A streptococci. Notably, DNase deletion mutants in this strain of bacteria exhibited increased susceptibility to neutrophil killing *in vitro,* and *in vivo* they demonstrated significantly less virulence in causing necrotizing fasciitis compared with the wild-type strain [75]. These data indicate the importance of NETs in limiting bacterial invasion and dissemination. Another form of NET killing avoidance utilized by bacteria is their expression of a polysaccharide capsule a feature often associated with increased virulence. This bacterial outer covering modification has been shown to significantly decrease pneumococci entrapment by NETs compared with the non-encapsulated strains [76]. Cell wall lipoteichoic acid modification on Gram-positive bacteria is a further adaptation, which enables evasion of NET killing. Some microorganisms appear to have evolved a relatively simple

avoidance method and stimulate release of relatively few NETs. Interestingly, our analysis of a panel of dental bacteria has shown that several strains may utilize this strategy of modulating NET release [71].

As early as 1974, Porschen & Sonntag reported DNAse activity in the endodontic infection-associated Gram negative bacteria *Fusobacterium nucleatum*, however, at the time the purpose of this enzymatic activity was not known [77]. Subsequently, others and we have shown the expression of DNase in several dental-disease relevant Gram-negative bacteria *in vitro* [78,79]. Notably, the expression of these bacterial DNases was found to be culture condition dependent, i.e. it could occur during either planktonic or solid state growth, and activity was either secreted or membrane bound. Studies also demonstrated that this bacterial DNase was able to degrade NETs *in vitro*. Clearly, within a biofilm, such as that occurring within root canals, expression by certain bacteria at particular stages of growth may confer benefits to the whole microbial community in terms of NET killing evasion. This virulence trait of the biofilm as a whole may then enable further propagation and dissemination of bacteria within the endodontic tissues. Interestingly, bacterial DNase expression may also be important in modifying the biofilm matrix to enable plaque development and maturation [80].

Endodontic disease associated bacteria may utilize several strategies to avoid NET entrapment, including DNase expression and modulated NET induction. In addition, bacteria such as *Enterococcus faecalis, Porphyromonas gingivalis and Porphyromonas intermedia*, have been shown to exhibit surface modifications including the presence of polysaccharide capsules with this phenotype strongly associating with root canal biofilm infections [81-83]. Whilst studies have not specifically correlated these bacterial outer surface modifications with NET evasion, there exists the potential that bacteria present in endodontic infections have evolved this type of virulence strategy to evade this form of innate immunity entrapment.

Host response to NETs

Whilst the NET response is aimed at protecting the host from invading bacteria, NETs have also been associated with auto-immune and -inflammatory pathology. Despite NET clearance being highly orchestrated, excess and aberrant NET release or clearance may provide a source of inflammatory and cytotoxic molecules. Notably, in the autoimmune disease Systemic lupus erythematosus (SLE), neutrophil-derived granular proteins associated with NETs, including MPO and PR3, can cause autoantibody responses in patients and NET clearance by sera from some patients is impeded [84]. This impaired removal of NETs has been attributed to the presence of DNase inhibitors in patients' sera or due to increased levels of anti-NET antibodies or complement factors, which may protect NETs from DNAse degradation [85]. Furthermore, delayed removal of NETs may provide a reservoir of PAD4

hyper-citrullinated proteins, which provides antigens and contributes to disease pathogenesis [86]. In the chronic inflammatory disease, rheumatoid arthritis (RA), neutrophils from RA patients have also been shown to generate elevated levels of NETs compared with healthy controls [87]. Subsequently, it has been demonstrated that the anti-citrullinated protein antibodies (ACPAs) within RA patient serum react with the histones in NETs, indicating that they may provide the immunogenic trigger for the vicious inflammatory cycle within patients' joints [88]. Interestingly, *P. gingivalis*, which is frequently found in endodontic infections, possesses its own PAD enzyme [89]. Conceptually, *P. gingivalis* PAD may citrullinate its own or host proteins locally and this may drive inflammation locally within the infected root canal network.

Recent studies in mouse models of lung disease have shown that NETs and their components cause significant damage to lung epithelial and endothelial cells. This effect was particularly evident in animals with an influenza virus and *Streptococcus pneumoniae* dual infection and was associated with compromised macrophage clearance of NET structures. Data indicated that the viral infection led to neutrophil priming, which were subsequently hyper-active in terms of NET release. These dual infected animals exhibited increased lung tissue damage, which was associated with exaggerated inflammation and damage to the alveolar-capillary barrier as compared with single viral or bacterial infected animals, which exhibited increased levels of survival [90,91]. While the immunogenic molecules described above could potentially contribute to this pathogenic process, a recent review has highlighted the potentially important role of histones in the pathobiology of several diseases [92]. While our preliminary studies (unpublished data) and those of others [55] have shown histones to have antibacterial activity, including killing of endodontic infection-associated bacteria, histones have also been reported to act as damage-associated molecular patterns (DAMPs) when they are released into the extracellular space.

There are five histone types responsible for the packaging of nuclear DNA and they are categorized into two groups: the core histones (H2A, H2B, H3, and H4) and linker histones (H1 and H5) [92]. Several stress associated mechanisms have been demonstrated to result in their release including apoptosis, necrosis and now NETosis. Subsequent cellular detection of extracellular histones by binding to receptors, such as TLR-2, -4, -9 and the inflammasome, in a range of cell types triggers activation of multiple signaling pathways, e.g. NF-KB, MAPK & Caspase-1, in a single or combined manner. This cellular activation leads to several processes including pro-inflammatory signaling, induction of cell death and platelet activation. Interestingly, high concentrations of serum histones are detected in animals or patients with cancer, inflammation, and infection, and inadequate clearance of this cellular debris by macrophages may lead to accumulation of these damage or disease markers. Indeed, extracellular histones have been considered mediators of several systemic inflammatory diseases and

sterile inflammation. Potentially, histones now represent a therapeutic target for many infectious and inflammatory disorders [92].

These published reports relating to the pro-inflammatory and cytotoxic nature of NETs and their components have driven us to explore their effects on pulpal cell populations. Our preliminary *in vitro* data are consistent with that seen in other diseases and indicate that single histones, such as H2A or combinations of histones, are cytotoxic to human pulp cells and can drive IL-8 release, which potentially could perpetuate the chronic inflammatory cycle within the pulpal tissue. Interestingly, the exposure of pulp cell cultures to DNA on its own was not able to exert these cellular effects (unpublished results). Further work is however required to validate these preliminary findings and determine their physiological relevance.

Concluding remarks

While the exact nature of NETs is still not completely agreed upon, in particular with regards to their nuclear or mitochondrial origin [93], the existing data supports NET-DNA release likely representing an important evolutionary and conserved antimicrobial mechanism in immunobiology. Indeed, studies of plant root-tip pathology have shown that the mucilaginous or 'slime-like' matrix encapsulating the plant root cap includes significant amounts of extracellular DNA, which inhibits fungal growth [94,95].

It is interesting to postulate that NETs and their components, such as histones, may provide novel prognostic markers for pulp pathologies. Indeed, the determination of their levels within diseased tissues, such as that of the infected pulp, could be exploited to target application of novel disease management strategies. It is also interesting to speculate that the epigenetic state of the histones released, including their level of citrullination, may affect their antimicrobial and auto-inflammatory properties. Such a concept could underpin the development of novel antibacterial therapeutic approaches as it is clear that infecting bacteria, such as *P. gingivalis*, aim to perpetuate chronic inflammation within the host tissue to increase nutrient availability and enable themselves and the biofilm to thrive. While some indirect data exist [96] which indicate the presence of NETs in infected pulpal tissue and a recent study has highlighted that extracellular DNA can be protected by dentin [97], there is now considerable scope for further research to characterize their roles in endodontic pathobiology.

ACKNOWLEDGMENTS

This work was supported by the School of Dentistry, University of Birmingham.

The authors deny any conflicts of interest related to this study.

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FIGURE LEGENDS

Figure 1. Schematic of aspects of the cellular and molecular processes involved in dentin-pulp complex immune response to infection. **(A)** Bacteria colonize and infect the wound, and are subsequently detected by tissue resident cells, including immune cells such as dendritic cells **(Bi)** and mast cells **(Bii)**. Vasoactive molecules and chemokines/cytokines are released to generate signals and gradients to enable further immune cell populations, such as neutrophils **(C)**, to be recruited from the blood stream. Subsequently neutrophils mediate bacterial killing **(D)** using mechanisms described in ***Figure 2***. The cycle of the inflammatory response continues until the infection is cleared. Macrophages will remove bacteria and cellular debris and drive resolution of inflammation and subsequently wound regenerative processes will be invoked to enable tissue healing.

Figure 2. Neutrophils use both intra- and extra-cellular killing mechanisms to eliminate pathogens. For intracellular killing, when neutrophils encounter bacteria, they phagocytose them and encapsulate them in phagosomes, prior to eliminating them using ROS or AMPs released from their granules. AMPs can also be released into the extracellular environment (degranulation) where they can directly kill bacteria. As described in detail within the main text body, NETs can also be released into the extracellular environment via a process termed NETosis. The core DNA of NETs is decorated with histones, plus AMPs and enzymes that are released from granules. NETs immobilize bacteria preventing their spread, localising them for killing with histones and other AMPs, as well as facilitating their phagocytosis.

Figure 3. **(A) (i)** ROS and **(ii)** NETs induced at 4 hours stimulated with heat-killed endodontic infection associated bacteria, *P. gingivalis* (MOI 1:100) and *F. nucleatum* (MOI 1:100), along with the PMA (50nm) positive control. Statistical significant differences from negative PBS control are shown with p-values *=0.05, **=0.01, ****=0.0001. N=4. **(A)(iii)** Example fluorescent image of NETs induced by PMA stimulation. Nuclei and extruded NETs are stained with Sytox Green. **(B)** Fluorescent images of **(i)** control (unstimulated) and **(ii)** *F. nucleatum*-induced NETs stained with Sytox green. **(iii)** Fluorescent image showing some *F. nucleatum* bacteria associating with a NET-DNA strand. All techniques for ROS and NET analysis imaging were performed as previously reported [65]. Scale bars are shown in micrographs.

Statement of Clinical Relevance (max 75 words)

Infection of the tooth's tissues elicits an inflammatory response and until the infection and inflammation are resolved then dentin and pulp repair mechanisms are impeded. Neutrophils combat the infection within the pulp and release Neutrophil Extracellular Traps (NETs) which encapsulate and kill bacteria. NET components however can be pro-inflammatory and cytotoxic. Components of NETs could serve as new prognostic markers and provide novel therapeutic targets to aid vital pulp therapy.

Fig. 1

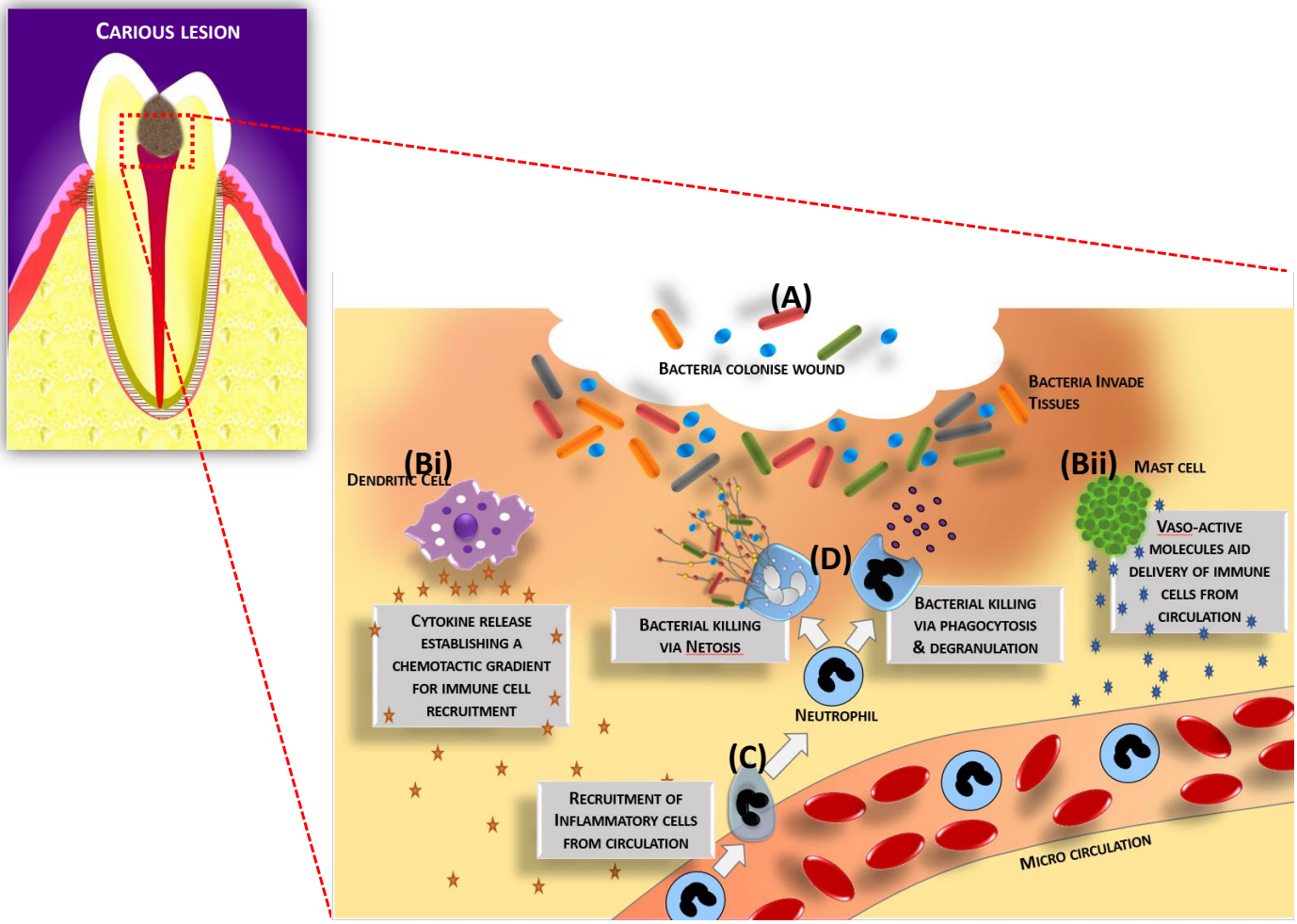

CARIOUS LESION
(A)
BACTERIA COLONISE WOUND
BACTERIA INVADE TISSUES
(Bi)
DENDRITIC CELL
CYTOKINE RELEASE ESTABLISHING A CHEMOTACTIC GRADIENT FOR IMMUNE CELL RECRUITMENT
(Bii)
MAST CELL
VASO-ACTIVE MOLECULES AID DELIVERY OF IMMUNE CELLS FROM CIRCULATION
(D)
BACTERIAL KILLING VIA NETOSIS
BACTERIAL KILLING VIA PHAGOCYTOSIS & DEGRANULATION
NEUTROPHIL
(C)
RECRUITMENT OF INFLAMMATORY CELLS FROM CIRCULATION
MICRO CIRCULATION

Fig. 2

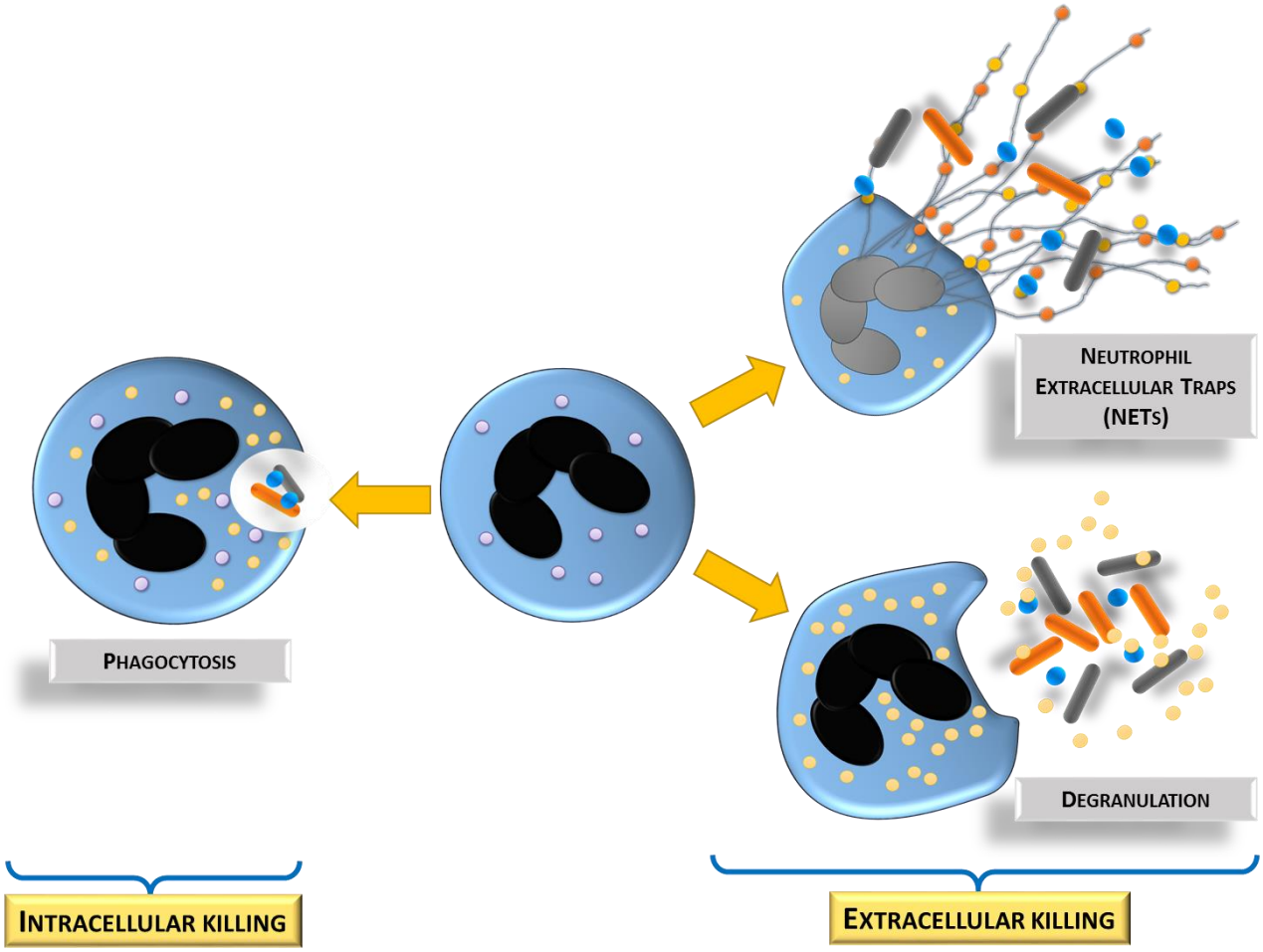
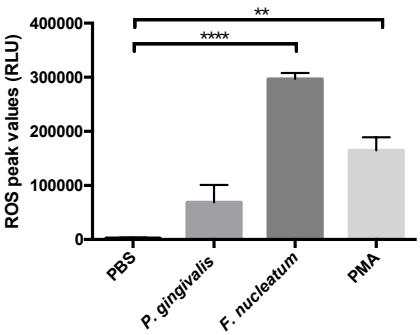

NEUTROPHIL EXTRACELLULAR TRAPS (NETS)
PHAGOCYTOSIS
DEGRANULATION
INTRACELLULAR KILLING
EXTRACELLULAR KILLING

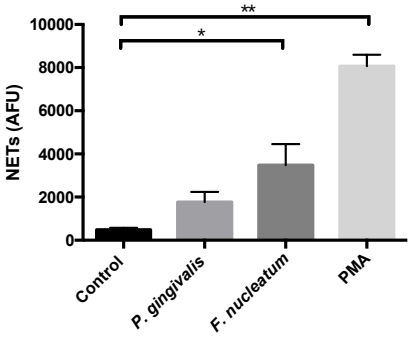
Fig. 3

(A)

(i)



(ii)



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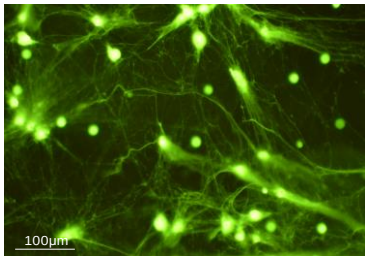
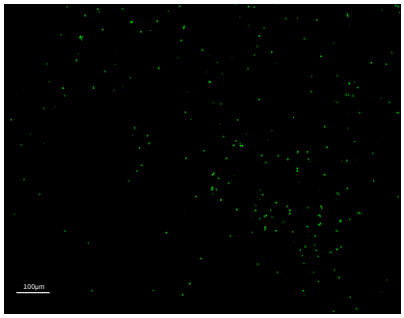
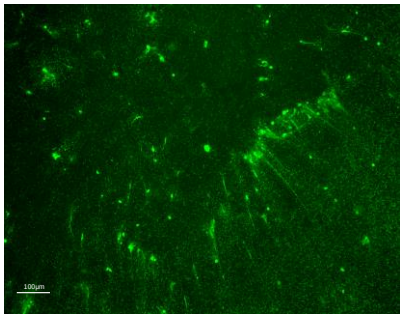


(B)

(i)



(ii)



(iii)