6 Current Understanding and Future Applications in Dentine-Pulp Complex Inflammation and Repair

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6.1 Introduction

Erupted teeth are covered by symbiotic microbial communities organised in biofilms, mainly composed of Gram-positive saprophytic bacteria. These biofilms adhere to the enamel surface and are normally harmless to the tooth; however, increased bacterial metabolic activity in response to a sugar-rich environment results in the release of acids that progressively demineralise the enamel (Hamilton 2000; Farges et al. 2009). A carious lesion thus develops which is characterised by the formation of a cavity within which "cariogenic" bacteria grow and release additional acids deepening the lesion. The dentine subsequently becomes affected and demineralised by this activity of microorganisms, such as *Streptococci*, *Lactobacilli* and *Actinomyces*, that predominate the local Gram-positive microflora (Love and Jenkinson 2002). Proliferating intra-dentinal bacteria release by-products which diffuse down the dentinal tubules and towards the peripheral pulp. Concomitantly

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the demineralisation of the dentine matrix, due to the acidic environment, releases the bioactive molecules archived within it (Cooper et al. 2011). The recognition of the bacterial components initially by the odontoblasts at the pulp periphery is the trigger for local protective events including the production, by pulp cells, of antibacterial, immune, inflammatory and dentinogenic molecules. This activity aims to both limit the bacterial infection and block its progression to the pulp by the formation of tertiary dentine at the pulp-dentine interface. If the bacterial invasion remains unchecked, however, irreversible pulpitis will occur which will ultimately lead to pulp necrosis and infection within the tooth root canal system. Invading microorganisms will then disseminate into the periapical regions and trigger periapical disease (Love and Jenkinson 2002; Heyeraas and Berggreen 1999). These series of events result in important dental and supporting tissue damage, and ultimately the tooth may be lost following periodontal tissue destruction. If the early-stage dentine infection is clinically removed by the practitioner, the pulp inflammation should subside (Hahn and Liewehr 2007a) and tissue healing with tertiary dentine formation can occur (Lesot et al. 1994). The newly formed dentine will protect the pulp from further infection as well as from any restorative filling material placed. From a clinical standpoint, it is reasonable to speculate that the induction of tertiary dentine, by distancing the pulp from the affected dentine, will help protect the pulp, promote healing and maintain pulp vitality, thereby enhancing tooth longevity. The identification of the molecular and cellular mediators, which dampen the immune/inflammatory response, while stimulating tertiary dentine formation, and which may promote a return to pulp tissue homeostasis and health following bacterial infection resolution, has therapeutic potential (Farges et al. 2009, 2013; Cooper et al. 2014; Gaudin et al. 2015). Subsequently studies are underway aimed at obtaining a better understanding of the events that initiate and control the pulp's antibacterial-, immune- and dentinogenic-mediated defences to enable the development of novel treatments.

6.2 Early Stages of the Dentine-Pulp Complex's Host Defence Response

Due to their location and cellular processes penetrating into the dentinal tubules, odontoblasts are the first cells within the tooth to be encountered by the molecular components released by the invading pathogens (Durand et al. 2006; Veerayutthwilai et al. 2007). Pathogen recognition occurs via the detection of bacterial structures termed pathogen-associated molecular patterns (PAMPs), and these are sensed by a limited number of so-called pattern recognition receptors (PRRs). A key class of PRRs is the Toll-like receptor (TLR) family, which is essential for triggering the effector phase of the innate immune response (Fig. 6.1) (Beutler 2009; Kawai and Akira 2010; Kumar et al. 2011). TLR-2 and TLR-4 detect the Gram-positive and Gram-negative cell membrane components lipoteichoic acid (LTA) and lipopolysaccharide (LPS), respectively. They have been shown to be present on odontoblasts from healthy pulp, indicating the tissue is equipped to initially recognise early

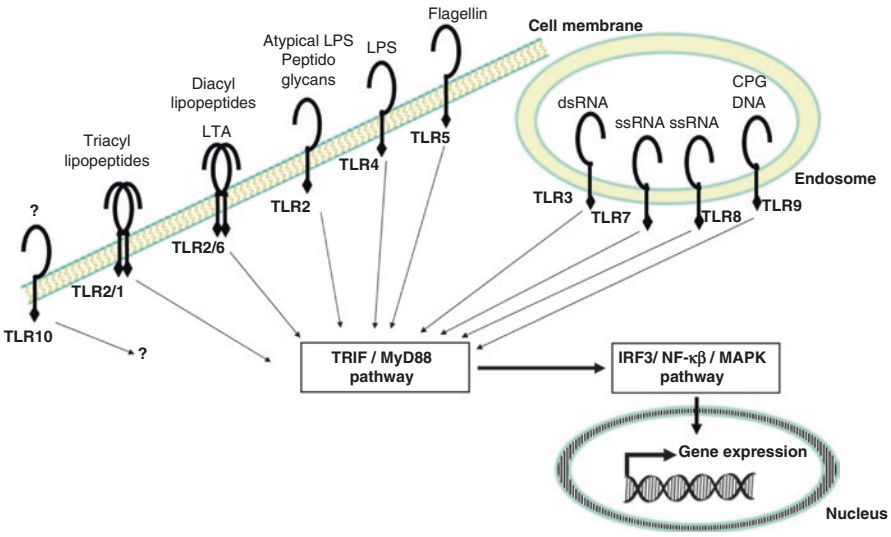

Fig. 6.1 Overview of Toll-like receptor (TLR) signalling pathways which sense bacterial components (examples indicated above each TLR). Pathogen recognition receptors (PRRs) are present on cells present in the dentine-pulp complex (as described in the main text body), and their binding of microbial components results in transcriptional activation of pro-inflammatory and dental repair-associated genes. TLRs comprise two components and form homo- or heterodimers which are located on the outer cell membrane or on endosomal membranes. The binding of the bacterial components initially results in activation of downstream intracellular signalling molecules including TRIF and MyD88. Activation of these molecules results in further IRF, NF-κB and MAPK signalling activation which culminates in nuclear translocation and transcription factor activation. The cytoplasmic PRRs of NOD (nucleotide-binding oligomerization domain)-1 (Gram positive) and NOD-2 (Gram negative) have also been reported as being present in dental cells, and they transduce signals via NF-κB, NALP3 (NACHT, LRR and pyrin domain-containing protein 3) and caspase-1 signalling. ? = the ligand and downstream signalling from TLR10 are not yet known. Abbreviations: IRF = IFN regulatory factor; MAPK = mitogen-activated protein kinase; MyD88 = myeloid differentiation primary response gene 88; NF-κB = nuclear factor-κB; TRIF = Toll/IL-1R (TIR) domain-containing adaptor protein inducing IFN-β

dentine infection (Veerayutthwilai et al. 2007; Jiang et al. 2006). TLR2 is reportedly upregulated in odontoblasts beneath caries lesions (Farges et al. 2009), indicating that these cells can adapt and potentially increase their sensitivity for pathogen recognition.

TLR activation results in upregulation of innate immune responses manifested by local release of antimicrobial agents and pro-inflammatory cytokines and chemokines which recruit and activate immune/inflammatory cells (Viola and Luster 2008; Turner et al. 2014). Notably, odontoblasts have been shown to express several antimicrobial agents, such as beta-defensins (BDs) and nitric oxide (NO). The BD family comprise of cationic, broad-spectrum antimicrobial peptides that elicit their killing mechanisms by forming channel-like micropores that disrupt membrane integrity and induce leakage of the microbial cell content (Pazgier et al. 2006; Sørensen et al. 2008; Semple and Dorin 2012; Mansour et al. 2014). In general

BD-1 is constitutively expressed, whereas BD-2, BD-3 and BD-4 are induced in tissue expression following microbial contact with the host. Several in vitro studies have now reported BD involvement in pulpal defence during caries. BD-2 has been shown to exert antibacterial activity against *Streptococcus mutans* and *Lactobacillus casei* (Shiba et al. 2003; Song et al. 2009; Lee and Baek 2012), while BD-3 is active against more mature biofilms containing *Actinomyces naeslundii*, *Lactobacillus salivarius*, *Streptococcus mutans* and *Enterococcus fæcalis* (Lee et al. 2013a). BD-2 can also feedback by autocrine and paracrine mechanisms to amplify the inflammatory response and can upregulate interleukin (IL)-6 and IL-8 in odontoblast-like cells in vitro (Dommisch et al. 2007). A positive feedback mechanism may exist between cytokines and BD-2 as its expression can be stimulated by the cytokines IL-1α and tumour necrosis factor (TNF)-α in cultured human dental pulp cells (Kim et al. 2010; Lee et al. 2011). The pro-inflammatory effects of BD-2 are also highlighted by its ability to chemoattract immature antigen-presenting dendritic cells (DCs), macrophages, CD4+ memory T cells and natural killer (NK) cells (Semple and Dorin 2012). Studies using a tooth organ culture model have shown that odontoblast BD-2 gene expression was not affected by TLR2 activation; however, BD-1 and BD-3 transcript levels were downregulated (Veerayutthwilai et al. 2007). Interestingly BD-2 gene expression was elevated following TLR4 activation. In vivo studies have also shown that odontoblasts in healthy tissue express BD-1 and BD-2 (Dommisch et al. 2005; Paris et al. 2009). Combined, these data indicate that BDs are differentially expressed in the pulp tissue and that there is a low level of constitutive expression of BDs by odontoblasts and other cells within the pulp protecting the tissue from infection. There is, however, a degree of controversy regarding expression levels of BDs in inflamed dental pulp. Initially BD-1 and BD-2 were reported to be decreased in irreversible pulpitis (Dommisch et al. 2007); however, more recent work has shown increases in BD-1 and BD-4 (but not BD-2 and BD-3) in pulp tissue at a similar stage of disease (Paris et al. 2009). Clearly further studies are required to better understand the role and regulation of BDs in dentine-pulp complex health and disease.

Reactive nitrogen species (RNS), such as NO, are potent antibacterial molecules. They are highly diffusible free radicals generated by the oxidative action of NO synthases, of which there are three isoforms, NOS1 (neuronal NOS), NOS2 (inducible NOS) and NOS3 (endothelial NOS), which produces NO from L-arginine. NOS1 and NOS3 are constitutively expressed in most healthy tissues; however, NOS2 can be induced following microbial challenge. NOS2 is mostly involved in host defence due to the relatively high micromolar range amounts of NO that it can generate over long time periods (hours to days) (Nathan 1992; Nussler and Billiar 1993; MacMicking et al. 1997; Coleman 2001; Guzik et al. 2003; Arthur and Ley 2013; Bogdan 2015). Notably, NOS2 was only detected at relatively low levels in healthy human pulp but was significantly upregulated in inflamed pulps (Law et al. 1999; Di Nardo Di Maio et al. 2004). Interestingly in an experimental rat incisor pulp model of inflammation, NOS2 activation also promoted an increase in neutrophil and macrophage influx (Kawanishi et al. 2004; Kawashima et al. 2005). This process may be mediated by the chemoattractant,

IL-8, as NO is known to stimulate its production in human pulp cells. Recent studies have also indicated that human odontoblasts constitutively release NO which might provide an important defence mechanism against *Streptococcus mutans*, and in inflamed tissue, its release is further mediated by NOS2 activation to combat the later stages of disease (Korkmaz et al. 2011; Min et al. 2008; Silva-Mendez et al. 1999; Farges et al. 2015).

Numerous in vitro studies have demonstrated the ability of odontoblasts to produce inflammatory cytokines and chemokines when exposed to PAMPs (Durand et al. 2006; Veerayutthwilai et al. 2007). Indeed odontoblast-like cells in vitro have been shown to be responsive to LTA via TLR2 detection resulting in upregulation of TLR2 itself as well as the nucleotide-binding oligomerization domain-containing protein 2 (NOD2), a cytosolic pattern recognition receptor (PRR). This exposure activated NF-κB and p38 mitogen-activated protein kinase (MAPK) signalling pathways (Fig. 6.1), inhibited dentinogenesis and promoted the production of several pro-inflammatory chemokines, including CCL2, CXCL1, CXCL2, CXCL8 (IL-8) and CXCL10 (Farges et al. 2009, 2011; Durand et al. 2006; Staquet et al. 2008; Keller et al. 2010, 2011). This chemokine "storm" will lead to chemoattraction and activation of a range of immune cells within the pulp. During the early stages of caries, immature DCs are initially attracted and accumulate at strategic sites beneath the lesion in readiness to capture foreign antigens. Subsequently there is also a progressive and sequential accumulation of T cells/lymphocytes, macrophages, neutrophils and B cells/lymphocytes in the pulp as the lesion and bacterial infection increase (Hahn and Liewehr 2007a; Farges et al. 2003; Jontell et al. 1998). Others have shown that the pleiotropic cytokine, IL-6, regulates many aspects of the local immune responses and is strongly upregulated by odontoblasts in vitro following TLR2 exposure (Farges et al. 2011; Hunter and Jones 2015; Nibali et al. 2012). IL-6 is critical to the differentiation of T helper (Th) 17 cells, while IL-6 inhibits regulatory T-cell (Treg) differentiation. Notably the main function of Tregs is to restrain excessive effector T-cell responses. IL-6 has also been shown to be important in promoting the secretion of acute-phase proteins such as LPS-binding protein (LBP) (Turner et al. 2014) as well as increasing vascular permeability to facilitate immune cell movement. It is therefore conceivable that odontoblast-derived IL-6 may modulate several functions in the infected pulp including oedema formation in response to bacterial infection.

IL-10 is a modulatory cytokine previously shown to be upregulated in bacterial infected pulps, and it has also been shown to be upregulated in odontoblast-like cells in vitro upon TLR2 engagement (Farges et al. 2011; Lee et al. 2012). IL-10 acts as an immunosuppressive cytokine, and for example, is able to decrease the production of the pro-inflammatory cytokines IL-6 and IL-8 (Li and Flavell 2008) and inhibit the Th1 and Th2 immune responses while promoting Treg differentiation (Saraiva and O'Garra 2010; Kaji et al. 2010). Subsequently it has been proposed that, as odontoblasts express this molecule, they therefore have the ability to molecularly limit local tissue inflammatory intensity (Farges et al. 2011).

Recent work studying the role of LBP has shown that this acute-phase protein attenuates pro-inflammatory cytokine production by preventing the binding to host

cells of several bacterial cell wall components including LPS, LTA, lipopeptides and peptidoglycan (Lee et al. 2012). In vitro, LBP has been shown to be upregulated in TLR2-activated odontoblast-like cells (Carrouel et al. 2013) and is also elevated in bacteria-challenged inflamed pulps. Potentially this molecule might decrease the effects of bacterial components, thereby also enabling modulation of the local dental immune response.

In summary, several studies demonstrate that odontoblasts are able to detect microorganisms and then respond to defend the tooth using their antibacterial arsenal (e.g. BDs, NO) and by signalling (e.g. chemokines, cytokines) to alert immune cells to combat the infection. This response is analogous to that found in other bodily tissues which become infected.

6.3 Immune Cell Responses in the Pulp

Clinically the removal of the tooth's decayed and infected hard tissues aims to lead to decreased pulpal inflammation, tissue healing and homeostatic recovery. Similar to other peripheral tissues, the healthy dental pulp is known to contain sentinel immune cells, including macrophages, DCs and T cells which undertake immunosurveillance (Farges et al. 2003; Jontell et al. 1998; Mangkornkarn et al. 1991; Izumi et al. 1995). Recent work has shown that leukocytes comprise ~1% of the total cell population in non-erupted healthy human third molar pulps (Gaudin et al. 2015). Following infection these numbers significantly increase due to chemoattraction from the circulatory system. Neutrophils are recruited in high numbers to the infected pulp, where they aim to combat the bacteria via intra- and extracellular killing mechanisms. In addition there is an increase in monocyte numbers which differentiate into macrophages (Cooper et al. 2011, 2014, 2010; Hahn and Liewehr 2007a, b; Jontell et al. 1998; Okiji et al. 1997). Bacterial phagocytosis by the macrophages activates T cells which trigger an adaptive immune response in association with DCs. Immature DCs are also attracted for bacterial antigen capture by odontoblast-derived chemokines (Hahn and Liewehr 2007a; Durand et al. 2006; Staquet et al. 2008; Jontell et al. 1998). Antigen uptake activates the maturation of DCs which then express a range of cytokines that regulate both the innate and adaptive immune responses. The latter is activated following DC migration to regional lymph nodes where they present antigens to and activate naive CD4+ T cells. The activated naive CD4+ T cells subsequently differentiate into effector CD4+ T helper cells (including Th1, Th2 or Th17 subsets) or induced regulatory T cells (Tregs) (Onoe et al. 2007). Recent analysis of T-cell populations in healthy human dental pulp has indicated that cytotoxic CD8+ T cells represent ~21% of total leukocytes and CD4+ T cells represent ~11%, with DCs ~4% of the leukocyte population (Gaudin et al. 2015). There is a progressive and sequential accumulation of CD4+ and CD8+ T cells as pulpal disease progresses (Cooper et al. 2011; Jontell et al. 1998; Okiji et al. 1997). Our knowledge of the mechanisms that regulate Th1, Th2 or Th17 responses in the pulp is essential to better understand pulp pathogenesis; however, currently data is minimal. Interestingly a recent study has

proposed that the control of IL-6 activity by MMP-3 could decrease Th2 and Th17 responses which may enable pulp regenerative events (Eba et al. 2012). NK cells have recently been identified in rat molar and incisor pulps, and they have also been shown to contribute to ~2.5% of the leukocyte population in healthy human pulps (Gaudin et al. 2015; Kawashima et al. 2006; Renard et al. 2016). Natural killer T (NKT) cells have also been detected in healthy rat pulp (Eba et al. 2012), and these cells play a major developmental role in Th1 versus Th2 immune responses (Kawashima et al. 2006). B cells are also reportedly present in healthy pulp tissue with their numbers significantly increasing during disease progression (Cooper et al. 2011; Gaudin et al. 2015; Hahn and Liewehr 2007b; Renard et al. 2016).

It is important to limit damage to the pulp that can occur collaterally by the complex immune cell mechanisms which are attempting to eliminate the microbial infection. Regulatory immune cells, such as Tol-DCs, may play a major role in this process (Tanoue et al. 2010; Banchereau and Steinman 1998). Notably they induce central and peripheral tolerance through different cellular and molecular mechanisms including T-cell depletion or anergy, induced Treg 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-β, indoleamine 2,3-dioxygenase (IDO), IL-27 and NO (Morelli and Thomson 2007; Li and Shi 2015). Tregs express molecules that inhibit or suppress the effector T-cell and Th cell responses. Interestingly, Tregs were identified in healthy human dental pulp (Gaudin et al. 2015), and a relatively large numbers of Tregs have recently been reported in severely inflamed human pulps (Bruno et al. 2010). Furthermore within healthy human pulp, there is also now evidence for the presence of a specific subset of immunoregulatory DCs which express HO-1 and protect cells against inflammatory and oxidative stress (Gaudin et al. 2015; Bruno et al. 2010). In addition myeloid-derived suppressor cells (MDSCs) which regulate immune responses have also been identified in healthy pulp (Gabrilovich and Nagaraj 2009; Dugast et al. 2008; Drujont et al. 2014). Notably the heterogeneous population of MDSCs can be expanded by exposure to bacterial components, such as LPS, and these regulate alloreactive T cells via HO-1 and IL-10 secretion (De Wilde et al. 2009). In an experimental rat incisor pulp model of reversible inflammation induced by LPS, an accumulation of an MDSC-enriched population and an increase of the expression of HO-1 and IL-10 were observed (Renard et al. 2016).

The healthy dental pulp is well equipped to detect and subsequently mount an efficient and effective immune response against invading bacteria. The range of resident leukocytes is much broader in healthy pulp than previously understood, and the immune and inflammatory response to the invading pathogens is complex. As the disease progresses, a range of immune cells are recruited from the circulatory system and these mature to reinforce the tissue's defence potential. Further work to better understand the pulp's cellular inflammatory response is warranted to enable development of novel immuno-therapeutics which could be exploited by the dental practitioner.

6.4 Interplay Between Pulp Inflammation and Healing

The immune and healing/repair responses within the tooth tissue are intimately associated. Indeed if possible, the tooth initially upregulates its dentinogenic responses to "wall off" any invading bacteria; if this first line of defence is overwhelmed however, the host's classical immune-inflammatory response is invoked to combat the bacterial invasion. Postnatal repair mechanisms within the dentine-pulp complex are well described and resemble tooth developmental processes in which progenitor cells in the dental papilla are molecularly signalled to differentiation into odontoblasts. During primary dentinogenesis, these newly formed odontoblasts secrete predentine which matures into dentine. In this cyclical process of dentine deposition, the mature odontoblasts continue to communicate with the dentine via their cellular processes which extend into the tubules. Subsequently bioactive molecules secreted by the odontoblast become fossilised within the dentine during its development (Jernvall and Thesleff 2000). The release of these dentine entombed signalling molecules later in life results in cellular events which modulate tooth tissue repair.

Primary dentinogenesis is reported to occur at a rate of ~4 μm/day of dentine deposition, while secondary dentinogenesis (which occurs throughout life after tooth root formation) decreases to a rate of ~0.4 μm/day (Nanci 2003). Tertiary dentinogenesis results in new dentine formation which distances and protects the surviving pulp from potential invading bacteria and is the tooth's natural wound healing response. Two distinct tertiary dentinogenic processes have been described (Fig. 6.2). Following relatively mild dental injury such as during early-stage caries, the primary odontoblasts become reactivated and secrete a reactionary dentine which has tubular continuity with the primary and secondary dentine. A greater injurious challenge, however, such as that occurring during a rapidly progressing carious lesion, results in primary odontoblast cell death beneath the lesion (Bjørndal 2008; Bjørndal and Darvann 1999). This cell death is potentially a result of bacterial toxins, components released from the demineralised dentine and/or local release of high levels of pro-inflammatory mediators. If, however, local conditions become conducive, for example, if the infection is clinically controlled or becomes arrested, stem/progenitor cells either within the pulp or ones more distant from it are recruited to the site of injury and differentiate into odontoblast-like cells. The tertiary dentine formed by these cells occurs at a similar rate of deposition to that of primary dentinogenesis, and clinically this can result in dentine bridge formation (Smith et al. 1995).

These two tertiary dentinogenic processes differ in their complexity. Reactionary dentinogenesis is comparatively simple and requires only upregulation of existing odontoblast activity, whereas reparative dentinogenesis involves several processes including progenitor cell homing, proliferation, differentiation and upregulation of dentine synthesis (Fig. 6.2) (Fitzgerald et al. 1990; Magloire et al. 1996). The source of the signalling molecules necessary for both these processes is derived from the bacterial acid demineralised dentine substrate (Smith et al. 1995, 2012; Simon et al. 2011). This molecular release due to the hard tissue breakdown enables odontoblasts and progenitor cells to detect and positively respond to the dental tissue

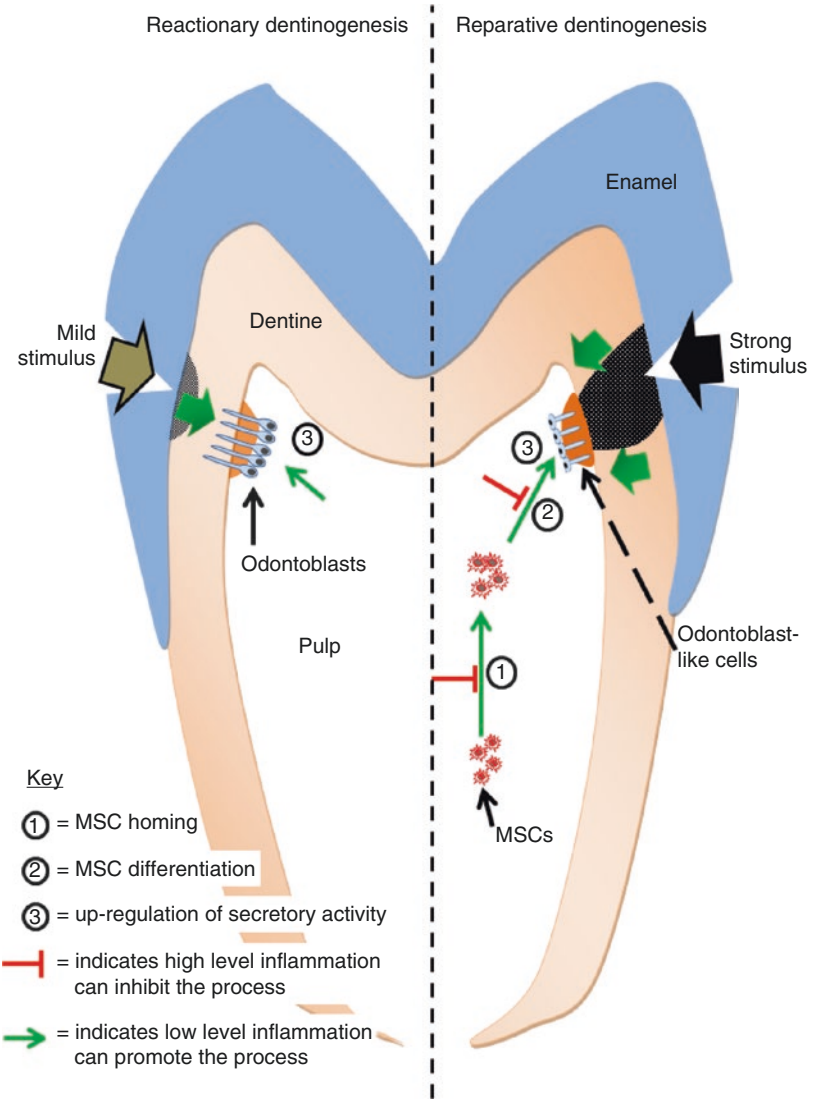

Fig. 6.2 The relationship between inflammation and the tertiary dentinogenic events of reactionary and reparative dentinogenesis. Low-level acute inflammation has the potential to stimulate reactionary dentinogenic events by upregulation of surviving odontoblast's secretory activity. Increased persistent and intense inflammation, such as during a rapidly progressive carious lesion, has the potential to lead to odontoblast death. If the infection is arrested, such as following clinical intervention using restoratives such as calcium hydroxide and MTA, the subsequent low-level resolving inflammation has the potential to signal MSC homing, differentiation and upregulation of their secretory activity. However if high-level inflammation ensues, the relatively high levels of inflammatory mediators may inhibit these MSC tissue repair-associated processes. MSC = mesenchymal stem cell, e.g. dental pulp stem cell (DPSC). Solid green arrows with gradients indicate release of dentine matrix components which signal tertiary dentinogenic events

damage. It is likely that it is the extent of the damage which drives the dentinogenic repair pathway activated. Notably not only do carious bacterial acids release the dentine's bioactive molecules, but certain restorative materials, such as calcium hydroxide and mineral trioxide aggregate, also do this. Furthermore it is now evident that a variety of mediators present during the inflammatory response are also able to signal tertiary dentinogenic events. A fine balance therefore exists between the levels of signalling molecules and their temporality in determining the nature of the tissue response.

As the carious infection progresses towards the pulpal core, the markers of the inflammatory process concomitantly increase including elevated levels of cytokines and immune cells (Hahn and Liewehr 2007b; Hahn et al. 1989; McLachlan et al. 2004). These cytokines exhibit a range of functions including regulation of lymphocyte recruitment, extravasation, activation, differentiation and antibody production. In the pulp, the roles of cytokines such as IL-1α, IL1-β as IL-4, IL-6, IL-8, IL-10 and TNF-α are well described in orchestrating the immune response (McLachlan et al. 2004; Hosoya et al. 1996; Matsuo et al. 1994; Pezelj-Ribaric et al. 2002; Lara et al. 2003; Dinarello 1984; Smith et al. 1980; Silva et al. 2004; Hahn et al. 2000; Barkhordar et al. 1999; Guo et al. 2000). Indeed, we have also reported significantly elevated levels at both the transcript and protein levels for a range of pro-inflammatory mediators, including S100 proteins, in carious diseased pulpal tissue. In addition, cytokines released from the demineralised dentine add to the complex milieu (Cooper et al. 2010; McLachlan et al. 2004). It is likely that not until the levels of these cytokines return to homeostatic ones then the chronic inflammation will persist within the tooth.

The inflammation that occurs within the tooth is double-edged as while it ultimately aims to kill invading bacteria, collateral host tissue damage can occur as a result of immune cell extravasation and antimicrobial activity. In particular it is well described that neutrophils release degradative enzymes, such as matrix metalloproteinases (MMPs), to enable their migration through the soft tissue matrix as well as generate reactive oxygen species (ROS) for extracellular antimicrobial killing. Notably, the ROS released can cause significant collateral tissue damage as well as stimulating further cytokine release via key pro-inflammatory intracellular signalling regulated by the p38 MAPK and NF-κB pathways (Veerayutthwilai et al. 2007; Simon et al. 2010; Fiers et al. 1999; Guha and Mackman 2001; Hagemann and Blank 2001). Notably while these signalling pathways are central to regulating the inflammatory response, they are also known to signal tissue repair events. More recently extracellular traps derived from neutrophils (NETs) have been described as a host antimicrobial mechanism. In this cell death process, termed NETosis, neutrophilic nuclear DNA is extruded via ROS-mediated pathways. The DNA fibres released are decorated with antimicrobial proteins derived from neutrophilic granules which aim to limit the spread of bacteria as well as cause their cell death. Our work in this area (Cooper et al. 2017) has indicated that NET release, while aimed at protecting the host, could have serious deleterious effects on the pulp as it may exacerbate the local inflammatory response as well as induce stem cell death.

It is now becoming apparent that persistent pulpal inflammatory processes impede reparative events, and the accepted paradigm is that pulp healing can only occur after removal of bacteria and significant dampening of the inflammatory process (Bergenholtz 1981; Rutherford and Gu 2000; Baumgardner and Sulfaro 2001). Some of the most significant evidence that infection and inflammation control are necessary to enable healing is derived from classical animal studies. Indeed data has demonstrated that dental tissue healing/repair was apparent only in artificial cavities made in germ-free mice compared with those that were infected and subsequently had inflamed pulps (Inoue and Shimono 1992). Further evidence regarding the effect of inflammation on repair is derived from in vitro studies that demonstrate the biphasic effects of pro-inflammatory mediators. At relatively low levels, these molecules, such as TNF-α and TGF-β and also ROS and LPS, can stimulate repair-associated events in dental cells, while at higher levels, such as during persistent inflammation, they cause cell death. Other work has also shown that stem cell differentiation processes are directly impeded by several pro-inflammatory signalling molecules (Lara et al. 2003; Simon et al. 2010; Smith et al. 2005; He et al. 2005, 2015; Pevsner-Fischer et al. 2007; Chang et al. 2005; Goldberg et al. 2008; Paula-Silva et al. 2009; Wang et al. 2015, 2014; Feng et al. 2013; Lee et al. 2006; Saito et al. 2011).

Further evidence of the link between inflammation and repair is evident from data demonstrating receptor sharing in immune and stem cell populations. The CXC chemokine receptor 4 (CXCR4) is expressed on both cell types (Murdoch 2000; Miller et al. 2008), and along with its ligand, stromal cell-derived factor-1 (SDF-1)/CXCL12, they have been shown to be present within the dentine-pulp complex and are upregulated during dental caries (Jiang et al. 2008a, b). There appears to be a logical explanation for the sharing of this chemotactic receptor by these cell types as infected and damaged tissues need to appropriately modulate the recruitment of both immune and stem cells to injury sites (About and Mitsiadis 2001). Subsequently, the determination as to which of these two cell types gets preferentially recruited appears to be locally regulated. Indeed studies have demonstrated that cytokines modulate the stem cell surface expression of CXCR4 with relatively high levels of pro-inflammatory mediators abrogating CXCR4-expressing stem cell activity at sites where inflammatory cell recruitment predominates (Murdoch 2000).

Differences in the number of steps involved in the two tertiary dentinogenic responses described mean that local tissue inflammation can exert differing effects (Fig. 6.2) (Cooper et al. 2010). Indeed, in reparative dentinogenesis, there is the opportunity for inflammatory modulation at the cell homing, differentiation and secretory stages, whereas during reactionary dentinogenesis, the inflammatory response can lead to upregulation of the odontoblast secretory activity or it may contribute to driving odontoblast death (Fig. 6.3). Potentially it is acute or low levels of these inflammatory signals that are necessary to signal repair responses, while higher chronic levels impede tissue repair and favour signalling of immune cell-related events. This interplay between the inflammatory and reparative responses would appear necessary and pragmatic as protecting the pulp with de novo dentine formation while it is under significant attack from infection, and its own

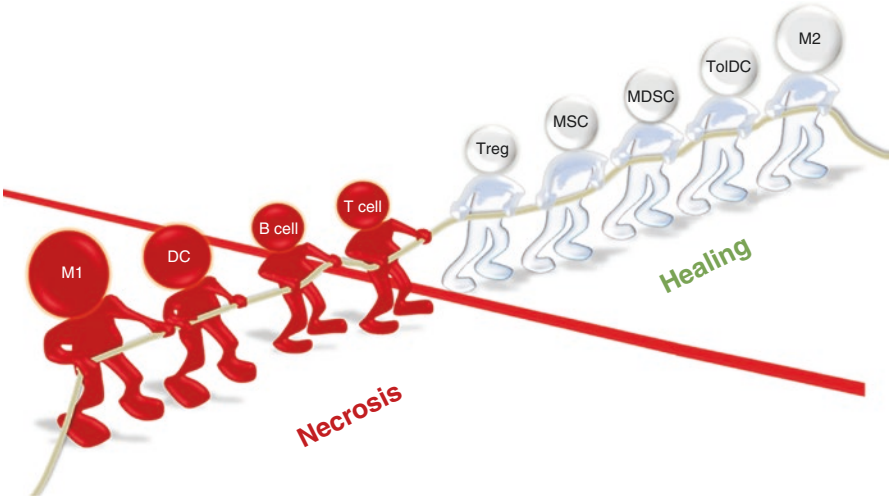

Fig. 6.3 Illustration showing the opposition between immune cells which promote cell and tissue necrosis when inflammation is excessive and those which dampen the immune response and promote healing in the dental pulp tissue (T cell, lymphocyte T helper and T cytotoxic; B cell, B lymphocyte and plasmocytes; DC, dendritic cell; M1, macrophage type 1; Treg, regulatory T cell; MSC, mesenchymal stem cell; MDSC, myeloid-derived suppressor cell; Tol-DC, tolerogenic dendritic cells; M2, macrophage type 2)

inflammatory response, would not be energetically efficient. Combined, the information presented here further support the notion that the modulation of the magnitude as well as temporospatial nature of the inflammatory response is central to determining tissue healing.

6.5 Tissue Inflammation and Healing: Translational Opportunities

Clinical observations following the application of pulp capping agents such as calcium hydroxide and mineral trioxide aggregate (MTA) provide further support for inflammatory events preceding dental tissue repair. These restorative agents are known to enable pulp healing beneath the site of application. Prior to tissue healing in the form of a dentine bridge, pulp tissue inflammatory events are routinely reported (Nair et al. 2008). While calcium hydroxide has been used clinically for over 60 years (Hermann 1930; Schröder 1985; Kardos et al. 1998; Goldberg et al. 2003), its mechanism of action for induction of reparative dentinogenesis still remains unclear. Its beneficial effects have however been attributed to local tissue irritation due to its elevated pH which causes cellular necrosis beneath the site of placement (Kardos et al. 1998; Schröder and Granath 1971; Stanley 2002). Hence, this tissue irritation has been cited as the principal mechanism of action which subsequently leads to the stimulation of an acute sterile inflammatory response

(Brentano et al. 2005; Luheshi et al. 2009; Acosta-Pérez et al. 2008; Magalhães-Santos and Andrade 2005). Furthermore MTA has been shown to stimulate cytokine release, including IL-1α, IL-1β, IL-2, IL-6 and IL-8 expression, from odontoblasts and osteoblasts, and this mild and acute material-induced inflammatory response may also contribute to clinical repair (Huang et al. 2005; Mitchell et al. 1999; Koh et al. 1998). Other studies have reported that the beneficial effects enabled by these restoratives are attributable to their ability to sterilise the site of infection while releasing bioactive components from the dentine (Graham et al. 2006; Tomson et al. 2007). It is therefore likely that several properties of these restoratives are important in generating a locally conducive environment to enable reparative dentinogenesis.

To gain a better understanding of the molecular response of the pulp tissue following carious destruction of enamel and dentine, high-throughput transcriptional profiling using diseased and healthy pulp tissue has been performed. These studies have indicated that the predominant tissue processes detected related to inflammation and there was minimal evidence of repair-associated molecular events (McLachlan et al. 2005). Differential expression of several molecules previously not associated with dental disease were identified, and one particular molecule, adrenomedullin (ADM), provided a candidate modulator for both inflammation and repair. This pleiotropic cytokine has reported antibacterial and immunomodulatory activities, as well as being able to promote angiogenesis and mineralised tissue repair (Zudaire et al. 2006; Montuenga et al. 1997; Ishii et al. 2005; Cornish et al. 1997). Our own studies subsequently demonstrated that ADM can exert similar effects within the dental tissues and that it was archived within the dentine during primary dentinogenesis (Musson et al. 2010). Mining of high-throughput transcriptional data obtained from well-characterised clinical samples has the potential to facilitate our understanding of the link between inflammation and regeneration and identify novel molecular targets for clinical exploitation.

Cell therapy approaches for dental disease are also being considered via the direct action of mesenchymal stem cells (MSCs) or indirectly via their secretome ability to modulate inflammation and promote dental tissue repair. Reported MSC immunomodulatory effects include:

- Inhibition of immune cell proliferation
- Inhibition of cytokine/antibody secretion
- Inhibition of immune cell maturation
- Inhibition of antigen presentation by T cells, B cells, NK cells and DCs (De Miguel et al. 2012; Leprince et al. 2012; Tomic et al. 2011)

Furthermore the direct cell-to-cell contact between stem and immune cells elicits MSC secretion of soluble factors such as TGF-β1 and IDO which have known anti-inflammatory effects. In addition, MSCs in dental pulp express TLR10 (Karim et al. 2016). The role of TLR10 is not well defined, but it appears to act as an inhibitory receptor, with suppressive effects (Oosting et al. 2014). Further work characterising the role of MSCs in inflamed dental tissues and their secreted components may enable development of novel cell therapy approaches.

Therapeutic modulators of inflammation have the potential to be used adjunctively, in particular along with disinfection regimes, to facilitate the healing response and potentially aid restoration longevity. Recent work has reported that dental resin restorative procedures supplemented with antioxidants, such as *N*-acetyl-cysteine (NAC), provide protection to pulpal cells from ROS generated following resin placement. Interestingly, NAC may also limit the activation of the key ROS-activated NF-κB pro-inflammatory pathway (Yamada et al. 2008), and this may minimise tissue inflammation and subsequently create a more conducive environment for healing. Indeed, other work has demonstrated the importance of the modulation of both ROS and RNS to enable repair. It has recently been demonstrated that the anti-inflammatory mechanism of exogenously applied PPARγ in human dental pulp cells was likely due to the removal of both NO and ROS. This application resulted in the suppression of both the NF-κB and extracellular signal-regulated kinase (ERK)1/2 signalling pathways (Kim et al. 2012). There have also been a significant number of studies assessing the anti-inflammatory effects in the pulp of other naturally derived compounds, for example, by pachymic acid, obtained from the mushroom *Formitopsis niagra*. 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 (Lee et al. 2013b).

Other areas where novel therapeutic anti-inflammatory opportunities exist include the relatively novel and exciting area of microRNA (miRNA) technologies. Recent work has shown the expression of these molecules with immunomodulatory capabilities in the pulp, and hence further work relating to their therapeutic application in the diseased pulp is being explored [(Zhong et al. 2012; Hui et al. 2017); also see Chap. 5]. We and others have been studying the application of low-level light therapy as a means to modulate inflammation and promote tissue repair. While this technology is more widely applied in the treatment of other diseases, there is significant potential for its application in dental disease, therefore further studies are warranted (Milward et al. 2014).

Conclusions

During a progressive carious infection, initially the odontoblasts detect the invading bacteria, and subsequently cells within the pulp core such as resident immune cells, fibroblasts, stem cells and endothelial cells further orchestrate the molecular response. Autocrine and paracrine signalling along with the bacterial acid-mediated release of bioactive molecules from the dentine amplifies the immune reaction which leads to a significant immune cell infiltrate. Until the infection is clinically resolved, the relatively high levels of pro-inflammatory mediators present in the local environment will impede healing events and retention of vital pulp. It is clear that sustained research in this area will result in the development of new diagnostics (see Chap. 2) and therapeutic approaches which will translate into clinical practice and benefit dental patients of the future.

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