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Investigation into toll like receptor mechanisms of action, in relation to *Porphyromonas gingivalis* in periodontitis

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Abstract

Periodontitis is a chronic inflammatory disease caused by bacterial invasion of the gingival epithelial cells within the oral cavity (Bostanci and Belibasakis, 2012). A key bacterium responsible for this is *Porphyromonas gingivalis (P. gingivalis),* a gram negative bacterium that evades the host immune system by cell infection. The chronic inflammation shown in periodontitis can cause tissue damage and bone degradation; this can lead to tooth loss (Hajishengallis et al 2011). Within this review *P.gingivalis* mechanisms of infection are outlined, along with its mediation of the immune system. Recognition by Toll like receptors is also investigated, and the literature on the manipulation of toll like receptors (TLRs) as mediators of inflammation is reviewed. The discovery of macrophage phenotypes and their expression of pro and anti-inflammatory cytokines are outlined, with reference to the potential use of human TLR7 inhibitor imiquimod, as a potential way of reducing inflammation within periodontitis.

Porphyromonas gingivalis

Porphyromonas gingivalis is a gram negative, biofilm forming, anaerobic bacterium, found within the oral cavity (Bostanci and Belibasakis, 2012). It is an opportunistic pathogen, and once it has infiltrated the cells it can cause acute inflammation of the gingival epithelial cells (Gingivitis). Chronic inflammation can lead to disease progression to periodontitis causing tissue necrosis and bone degradation (Hajishengallis *et al* 2011).

Attachment

Invasion of the gingival epithelial cells by *P.gingivalis* starts with the formation of a subgingival biofilm within the periodontal pocket (Kuboniwa and Lamont, 2010). This biofilm consists of multiple oral pathogens including: *Aggregatibacter actinomycetemcomitans, porphyromonas gingivalis,Tannerella forsythia, Prevotella intermedia, Prevotella nigrescens, Fusobacterium nucleatum and Parvimonasmicra* (Veloo *et al* 2012). The anaerobic conditions within the periodontal pocket, along with a close proximity to blood flow to provide heme, provides an optimal area for *P.gingivalis* colonisation, due to its need for heme and vitamin K for survival. This has been shown by a study into *P.gingivalis* concentrations in relation to periodontal pocket depth, measured via PCR. Results have shown that for every mm in depth the number *of P.gingivalis* cells increased 10-fold (Kawada *et al* 2004). *P.gingivalis* is found in 85% of chronic forms of periodontitis and thus is most associated with the disease (Yang *et al* 2004). It is considered to be a late coloniser and thus is closer to the gingival epithelium. The contact with the epithelium allows for Type 1 major fimbriae to bind to β integrin on host cell surfaces via focal adhesion kinase.

Infection of the gingival epithelial cell

After attachment, phosphorylation of c-jun N terminal Kinase (JNK) and down regulation of extracellular signal related kinase ERK1/2 allows for the bacterium to enter the gingival epithelial cell without detection. This is due to the down regulation of nuclear factor kappa B (NF-kB) inhibiting IL-8 secretion and apoptosis (Yilmaz *et al 2003).* The inhibition of IL-8 means that *P.gingivalis* can avoid neutrophil chemotaxis due to IL-8 being necessary for both neutrophil migration to the infected cell and adhesion (Hammond *et al* 1995). Degradation of IL-1β, IL-6 and sICAM has also been found within the infected gingival epithelial cell (Neil *et al* 2009). After internalisation the bacterium induces nucleation of the actin filaments on the host cell; this creates microspike-like protrusions and long stable microfilaments distributed throughout the cell, within 24 hours the actin and tubulin cytoskeleton is remodelled shown by fluorescence microscopy (Yilmaz *et al 2003).* Within the cell *P.gingivalis* can maintain viability and replicate (Belton *et al* 1999).

Survival in the host cell

Survival in host cells is maintained through the release of ATP-hydrolysing enzyme to suppress ATP-dependant apoptosis as well as activation of PI_3K/Akt pathways, JAK/Stat pathways, down regulation of caspase 3 activity and an up regulation in

Bcl-2 levels (Nakhjiri *et al* 2001). Dissemination to neighbour cells via actin bridges allows for the infection to spread without activating the immune response (Yilmaz *et al* 2006). *P.gingivalis* can also manipulate proliferation of the gingival epithelial cell, up regulating proliferation and thus increasing the depth of the periodontal cavity. This was confirmed via cytofluorimetry that showed proliferation was increased due to accelerated progression through S phase (Kuboniwa *et al* 2008).

Interaction with the immune system

Once the number of P.gingivalis bacterium has grown substantially, production of pro-inflammatory cytokines IL-8, IL-6, IL-1β and sICAM-1in the gingival epithelial cells occur. A reason for this may be that recruitment of the immune system to the area of infection increases angiogenesis, this in turn would bring the heme needed to sustain the larger amount of P*.gingivalis* bacterium (Bostanci and Belibasakis 2012, O'Brien-Simpson *et al* 2009).

P.gingivalis can also enter macrophages by the use of Complement receptor 3 (CR3)(CD11b/CD18). This is done through the use of inside out signalling to activate the ligand binding capacity of CR3, via interactions between fimbrial accessory Proteins and TLR2 (Figure 1). Once inside the cell P.gingivalis can inhibit IL-12p70 production consequently reducing T-cell activation (Wang *et al* 2007). Persistence of *P.gingivalis* within the macrophage may also be related to CR3, with wild type *P.gingivalis* showing a higher intracellular persistence compared to DAP type fimbriae containing *P.gingivalis*. A CR3 deficiency within macrophages causes an increase in intracellular killing of wild type *P.gingivalis*, consistent with the hypothesis that CR3-mediated internalisation increases intracellular persistence of *P.gingivalis*.

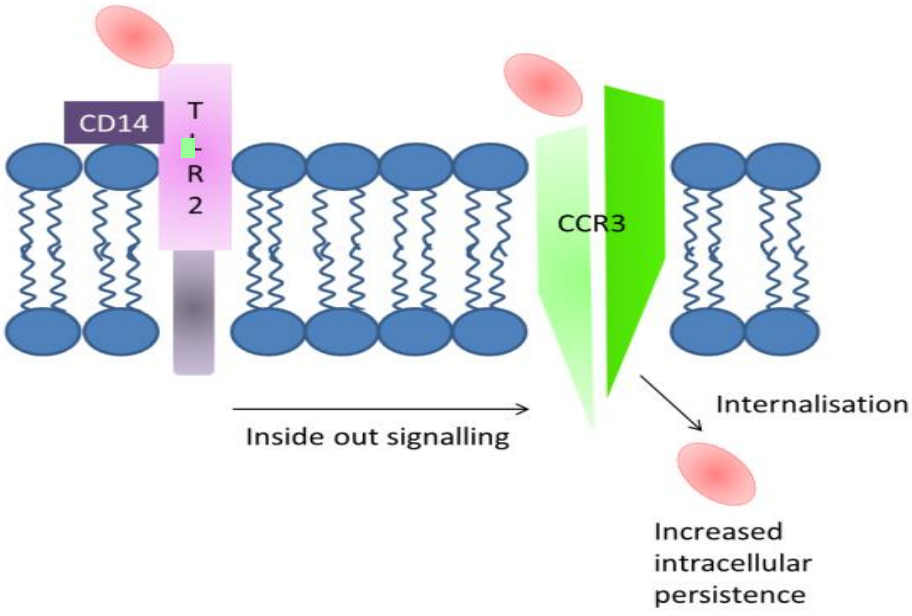

Figure 1: Modified from Wang *et al* 2007

Lipopolysaccharides (LPS) enable discrimination between commensal and pathogenic species through TLRs and CD14. *P.gingivalis* LPS is a pro-inflammatory cytokine stimulator, as well as a stimulator of bone resorption (Nishida *et al* 2001). However *P.gingivalis* is a slightly weaker cytokine stimulator than other LPS models. This may be due to differences in the O-antigen between *P.gingivalis* strains. These differences have a role in the acylation patterns and the receptor activating capacity of lipid A component. *P.gingivalis' s* lipid A, unlike most gram-negative bacterium, activates TLR2 instead of TLR4. In fact Darveau *et al* (2004) stated that the LPS may be an antagonist of TLR4. This is due to there being two different types of lipid A, tetra-acylated (TLR4 antagonist) and penta-acylated (TLR4 agonist). Dependant on heme concentrations, *P.gingivalis* can switch between the two types of lipid A, mediating the effect of host immune signalling (Al-Qutub *et al* 2006).

Investigation in to *P.gingivalis* LPS by Pulendran *et al* (2001) shows that *P.gingivalis* LPS activates $CD8\alpha^+$ and $CD8\alpha^-$ dendritic cells as well as murine peritoneal macrophages (Pulendran *et al* 2001). This results in the production of IL-6, and TNFα. However unlike *E.coli* LPS it does not induce IL-12 production. *P.gingivalis* LPS also stimulates metalloprpteinase-9 (MMP-9) expression in dendritic cells. MMP-9 is responsible for collagen cleavage for leukocyte migration towards the vasculature and lymphatic system. In a study with human monocyte derived dendritic cells it was shown that *P.gingivalis* increased expression of MMP-9, more than the tissue inhibitor of metalloproteinase-1 (TIMP-1) a regulator of MMP-9. Without regulation, excess amounts of MMP-9 cause large amounts of tissue destruction (Jotwani *et al* 2010).

The capsular polysaccharide (CPS) on *P.gingivalis* has the ability to generate systemic IgG antibody responses (Sims *et al* 2001), as well as resisting phagocytosis. Whereas cell surface cysteine proteinases called gingipains allow cleavage of CD2, CD4 and CD8 down regulating the immune response (Kitamura *et al* 2002). Gingipains also up regulate neutrophil protease activated receptor expression in neutrophils, Gingival epithelial cells, fibroblast and T-cells, crucial for chronic inflammation in periodontitis (Holzhausen *et al* 2010).

The combination of these virulence factors provides *P.gingivalis* with the tools to interact with the immune response, depending on extracellular factors. This along with our lack of understanding behind the molecular biology of many processes means that finding ways to effectively combat periodontitis are difficult. A way of combating periodontitis may be by reducing inflammation; this would decrease the amount of heme to the area as well as reduce tissue damage. This has been investigated with Rheumatoid arthritis, a chronic inflammatory disease of synovial joints (Sacre *et al* 2010).

Rheumatoid Arthritis

Rheumatoid arthritis (RA) is similar to periodontitis, as both are chronic inflammatory diseases. Also both result in the destruction of tissue and bone degradation due to persistent synthesis of pro-inflammatory cytokines, including TNFα and IL-6 as well as MMP (Midwood *et al* 2009). Both Periodontitis and RA share HLA-DRB1 alleles as well as sharing the same inflammatory pathways (Mikuls *et al* 2012).

Within RA TNFα has been highlighted as having a key role in inflammation (Davignon *et al* 2012). This is due to the blocking of TNFα causing a down regulation in other pro-inflammatory cytokines: IL-1β, IL-6 and IL-8, expressed by macrophages. It has also been suggested that *P.gingivalis* may play a role in early loss of tolerance to RA self-antigens (Mikuls *et al* 2012).

Anti-TNF reagents have been shown to reduce inflammation and bone erosion in some models; it works by reducing pro-inflammatory cytokines within the area and thus reducing inflammation. However anti-TNF treatments do not work for everyone (Fieldman *et al* 2010).

Toll like receptors

Toll like receptors (TLRs) are pattern recognition receptors (PRRs) that recognise molecular patterns on pathogens, known as pathogen-associated molecular patterns (PAMPs). Activation of periodontium cell TLRs by bacterial PAMPs lead to a release of pro-inflammatory cytokines, which elicit an inflammatory immune response that causes the damage to the surrounding tissue; as seen in periodontitis (Hans and Hans, 2011).

TLRs are part of a superfamily called the interleukin 1 Receptor/Toll-like receptor super-family. All members of the super family express Toll-IL-1 TIR domains which

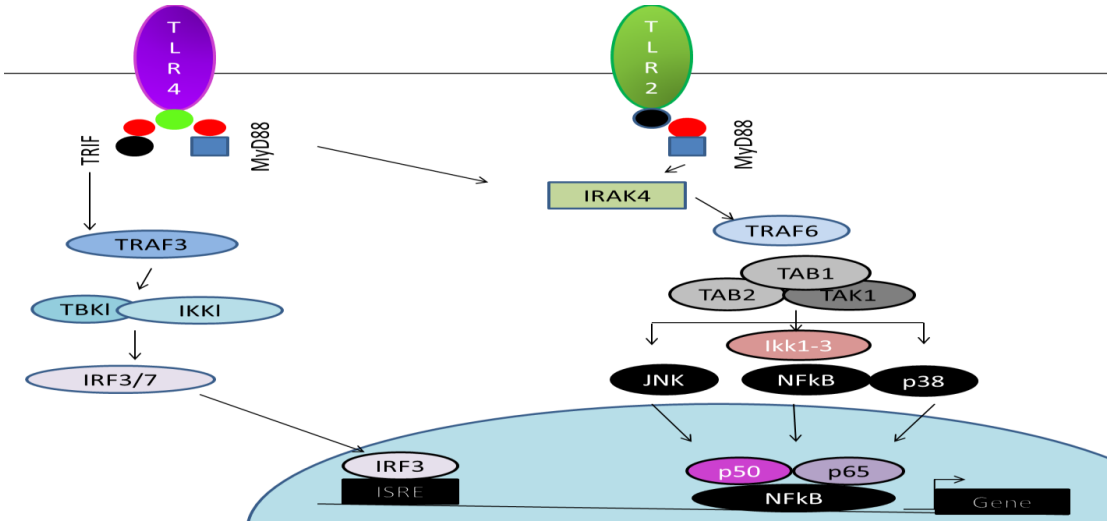

Figure 2: modified from Pennini et al 2012, showing the TLR 2 and 4 signalling pathways

are receptors for macrophage secreted interleukins (Hans and Hans 2011).

TLRs consist of a C-terminal cytoplasmic TIR domain, responsible for protein to protein interaction within innate immunity. This is connected to an N-terminal Leucine-rich repeat on the extracellular surface (LRR) that is responsible for Ligand binding and signalling. Within human cells TLRs 1, 2, 4, 5 and 6 are expressed on the cell surface membrane, whereas TLRs 3, 7, 8 and 9 are expressed intracellularly and specifically detect viral or bacterial nucleic acids. There are 10 TLRs that have been found to be expressed within human cells, however macrophages express TLRs 1, 2, 4, 5, 6, 7 and 8. *P.gingivalis* is known to stimulate TLRs 2 and 4, both receptors of LPS (Hans and Hans, 2011), yet other TLRs may also be activated. Research in to TLR activation of periodontal ligament and gingival fibroblast, after stimulation with P.gingivalis, showed a stronger induction of TLR1 and TLR7 (Scheres *et al* 2011). TLR1 induction may be due to TLR2 forming a heterodimer with the TLR1, and a reason for TLR7 induction may be that TLR7 plays a role in autophagy stimulation, in order to combat intracellular pathogens (Delgado *et al* 2008). These results however, may be different within a macrophage model. Activation of TLR2 is unusual, as it is normally associated with the recognition of gram-positive bacterial components through heterodimerizing with TLR1 or TLR6 (Pennini *et al* 2012).

All TLRs activate NF-$_k$B and MAP Kinase pathways (Figure 2). MyD88 is recruited in all but TLR4 and TLR3 signalling pathways, with TLR4 using both MyD88 dependant and independent signalling. (Pennini *et al* 2012) MyD88 dependant signalling recruits IL-1-receptor-associated kinases IRAK kinases: IRAK 1, 2 and 4 via death domains. IRAK kinases contribute to the myddosome, a critical multiprotein structure for TLR mediated signal transduction. Autophosphorylation of IRAK1 due to MyD88 phosphorylation of TRAK4 allows recruitment of ubiquitin ligase TRAF6 (Suzuki *et al* 2002). Ubiquitination of TRAF6 and TRAK1 enables interaction with IkB Kinase (IKK) complex. The IKK complex activates NF-kB transcription factor and MAP kinases ERK, p38 and JNK.(Brentano *et al* 2005) MyD88 independent signalling is via TRAM for TLR4 and TRIF dependant signalling for TLR3, resulting in pro-inflammatory cytokine production. (Pennini *et al* 2012, Brentano *et al* 2005).

TLR7 activation

TLR7 has been targeted for the regulation of inflammatory response within Rheumatoid arthritis (Alzabin *et al* 2012., Clanchy and Sacre, 2010). Mianserin was administered to arthritic mice daily after onset of disease. Results showed that inhibition of TLR7 decreased disease progression within mice models; However Mice model TLR7 is more like TLR8 within human models, and so may show differences when used in a human cell model. For example, Sacre *et al* (2008) investigates the inhibition of TLR8 to reduce TNF production in rheumatoid synovial membrane cultures. The use of Mianserin on human M-CSF macrophages showed a decrease in TNF production, inhibition via TLR8 was re-affirmed by the use of remiquimod (R-

848) a TLR7/8 agonist. The use of a TLR agonist as well as a TLR4 antagonist LPS, is a good way of ensuring activation through the desired TLR however finding an agonist specifically for TLR8 would help to enhance the results. Sacre *et al (2008),* found that inhibition of TLR8 could be achieved through the use of Imiquimod, a TLR7 agonist structurally similar to R-848. Also there was no effect with activation through LPS, showing that it did not act through inhibition of the high TNF producer TLR4. Further investigation could be in to whether or not production of TNF, after inhibition of TLR8 is effected by the macrophage phenotype.

Imiquimod has also been used in relation to IFN-β production (O'Brien *et al* 2009). The study showed an increase in IFN-β expression; this induces IL-10 anti-inflammatory cytokines thus working towards down regulating inflammation. The experiment was performed with 8 10-week old female mice. Imiquimod being a human and mouse TLR7 agonist may react differently in the human model. This is because the murine TLR7 behaves more like Human TLR8, which imiquimod has been suggested to inhibit in human macrophages (Sandre *et al 2008).*

Research in to TLR7 activation on the skin has shown to promote respiratory dendritic and natural killer cell accumulation *(*Hackstein *et al* 2012*).* An increase in anti-inflammatory cytokines was also observed, implying a reduction in inflammation and suppression of cytotoxic $CD8^+$T cells. This provides bases for further research in to murine TLR7, however as previously stated with other murine models there is still a need for investigation in to human TLR7/8 to provide any definitive results.

Macrophage autophagy

Macrophage autophagy and inflammation in atherosclerotic plaques, is shown to be induced by imiquimod (DeMeyer *et al* 2012). Imiquimod was chosen due to it being a TLR7 agonist; this allows specificity for targeting of the macrophage. Specificity is due to the smooth muscle cells (SMCs) not expressing TLR7 unlike the macrophage. A murine macrophage cell line was used as well as a human U937 cell line; this allows comparison between human and mouse cell lines. Also within the study TLR7 knock out mouse, bone marrow derived macrophages were used; this is good as it provides the information to determine if it is in fact TLR7 dependant. Before introduction to imiquimod, human atherosclerotic plaques were stained using immunohistochemical staining; the reaffirmation that the plaques still expressed TLR7 justifies the research. It was found that imiquimod caused cell death within 24 hours in macrophages and did not affect SMCs. Hyper phosphorylation of NF-kB in bone marrow derived macrophages, after introduction of imiquimod, induces inflammation via TLR7 expression, contradictory to previous studies where imiquimod is shown to down regulate inflammation in human models (Sandre *et al* 2008).

Autophagy allows for elimination of intracellular pathogens controlled by the recognition of PAMPs (Delgado *et al* 2008). Delgado *et al,* states that within

macrophages TLR7 was the most potent inducer of macrophage autophagy dependant on MyD88 expression. Autophagy induction is monitored by translocation of protein LC3 (ATg8) from the cytosol to newly formed autophagosomes. Within this study macrophages were transduced with fluorescent protein-LC3, 24 hours later Standard concentrations of PAMP were introduced as well as the inclusion of an autophagic control and a non-stimulus control. Results showed that TLR7 ligand produced the largest increase in LC3 puncta formation; this was then confirmed by the use of imiquimod as a TLR7 agonist at a concentration of 10µg/ml. MyD88 knockdown within cells demonstrated that TLR7 stimulated autophagy is MyD88 dependant.

The macrophage and its phenotypes

Macrophages play a major role in the innate immune response. Their ability to secrete a range of both pro-inflammatory and anti-inflammatory cytokines provides them with the ability to be key mediators in inflammation. Recent studies have shown there to be two phenotypes of macrophage; Classical activated (M1) and alternatively activated (M2), derived from monocytes after they are recruited in to the tissue via Chemokine signalling (Merry *et al* 2012). M1 macrophages are activated through LPS or IFNγ treatment; they're known for an increased release of pro-inflammatory cytokines such as IL-6 and TNFα, and are promoters of inflammation. Inducible nitric oxide synthase (iNOS) is also expressed within M1 macrophages; this enables the production of nitric oxide (NO) for microbicidal activity, which is not expressed in the M2 phenotype (Mege *et al* 2011). M2 macrophages are typically activated through the use of Th2 cytokines IL-4 and IL-13. The M2 macrophage expresses a higher concentration of anti-inflammatory cytokines like IL-10 and transforming growth factor- β (TGF-β), down-regulating pro-inflammatory cytokines in order to reduce inflammation (Merry *et al* 2012). These anti-inflammatory characteristics, along with their ability to help tissue regeneration via MMPs and growth factors, explain why M2 macrophages are more abundant within the resolving stages of inflammation (Weisser *et al* 2012., Merry *et al 2012*).

Skewing of macrophage phenotype has been suggested to be plastic, with cases of both M1 and M2 macrophages in the same area implying that the phenotypes may not be definite. Instead there may be a scale in which macrophages can move along dependant on the extracellular environment (Sica and Mantovani, 2012). Gemperle *et al (2012)* state that M2 macrophages have reduced amounts of formyl peptide receptor 1 (FPR1), stopping chemotaxis towards FPR1 ligand fMLF. This is however not true for M1 macrophages where chemotaxis was present. Further investigation into stimulation with a pro-inflammatory TLR4 agonist elevated chemotaxis, as well as FPR1 mRNA levels in M1 macrophages. This suggests that FPR1 expression promotes M1 activation and polarisation. The study could be taken further by investigating whether or not M2 macrophages can be stimulated into chemotaxis towards fMLF as they still express FPR1, even if it is in low amounts. This may be a

way of investigating the plasticity of macrophage phenotypes; if this is the case then it provides the possibility of using Macrophage phenotypes for the manipulation of inflammatory disease.

Proposal for future research

Activation of TLRs through *P.gingivalis* is not yet fully understood, with suggestions that it is not only recognised through TLRs 2 and 4 but TLR1 and 7 as well. Its avoidance of the innate and adaptive immunity through living within the host cells, as well as being able to mediate cytokine production, and in turn activation of immunity depending on what is most beneficial for the bacteria at the time, means that finding ways to fight periodontitis is a challenge. An area of exploitation may be with *P.gingivalis' s* need for heme in order to survive. Down regulation of inflammation in the gingiva, as well as an up regulation of M2 macrophages may lead to a reduction in the concentration of *P.gingivalis* cells due to a lack of heme for them all to survive. This reduction in cell count, combined with the reduction of tissue destruction from pro-inflammatory leukocytes, my provide relief from periodontitis.

So far imiquimod has been investigated for its TLR7 agonistic and TLR8 antagonistic properties within Human and murine cell bodies, yet there is no research on the effects of imiquimod on M1 and M2 human macrophages in relation to inflammatory cytokine production. If imiquimod induces a reduction in pro-inflammatory cytokines within M1 macrophages, like it has in RA models, then it may suggest a possibility in manipulating macrophage phenotypes for the control of chronic inflammation.

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