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The Potential of p38 MAPK Inhibitors to Modulate Periodontal Infections

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Abstract

Periodontal disease initiation and progression occurs as a consequence of the host immune inflammatory response to oral pathogens. The innate and acquired immune systems are critical for the proper immune response. LPS, an outer membrane constituent of periodontal pathogenic bacteria, stimulates the production of inflammatory cytokines IL- β , TNF α , IL-6 and RANKL either directly or indirectly. In LPS-stimulated cells, the induction of cytokine expression requires activation of several signaling pathways including the p38 MAPK pathway. This review will discuss the significance of the p38 MAPK pathway in periodontal disease progression and the potential therapeutic consequences of pharmacological antagonism of this pathway in the treatment of periodontal diseases.

Keywords

Periodontal diseases; p38 MAPK; p38 inhibitors; innate immunity; inflammation; bone loss; cytokine; experimental periodontitis

1. INTRODUCTION

Periodontal disease is a chronic infection of the periodontium, which encompasses both soft and mineralized tissues surrounding the teeth. Periodontal disease progression is associated with chronic inflammation of soft tissues, degradation of collagen fibers that attach the tooth to the gingiva and alveolar bone, as well as resorption of the alveolar bone itself. This can lead to tooth loss and there is some evidence indicating that this chronic infection may have negative systemic effects, including pre-term labor, imbalance of metabolic control in diabetics, complication of lower airway infections and aggravation of atherosclerosis [1,2].

Since the fundamental role of microorganisms in its etiology was scientifically demonstrated in the mid-60s, the research effort was long focused on identifying the pathogenic microorganisms and their virulence factors [3]. This search for culprit microorganisms was prompted by the fact that colonization of the oral cavity and presence of dental biofilm is normally associated with health, similarly to the colonization of the colon. Various therapeutic

strategies aimed at the microorganisms have been studied over the years, including local and systemic delivery of antimicrobial and antibiotic agents. The rationale for these therapeutic approaches is the fact that some species of microorganisms are considered to play prominent roles in periodontal disease based on their increased prevalence in the microbial flora associated diseased states.

Unique to this infection is the reality that the microorganisms associated with initiation and progression of periodontal disease are organized in a biofilm attached to the tooth structure, which places the microorganisms in intimate contact with the soft tissues without effectively invading the host. Even though bacterial invasion has been demonstrated in the periodontal tissues [4], most of the biofilm is located in proximity with the tooth surface, outside of the tissues. This fact significantly impairs the effectiveness of host immune defenses, as well as of therapeutic strategies utilizing antimicrobial chemical agents, to completely eradicate the infection [5].

For the past two decades, the host response to the bacterial challenge originating from the dental biofilm has been considered to play a major role on both initiation of the disease and on the tissue destruction associated with its progress [6]. The importance of host-microbial interactions is reinforced by epidemiological data indicating different susceptibilities to periodontal disease among individuals, in spite of the long-term presence of oral biofilm [7-9]. Other studies demonstrating increased susceptibility and greater severity of periodontal disease in individuals with impaired immune response due to systemic conditions also indicate the significance of the host response to the bacterial challenge [10,11].

Periodontal diseases provides unique situation to study microbial-host interactions. Over 500 different microbial species can be found in the oral biofilm [12], however only a few of those are associated with periodontal disease [13,14]. This recognition of pathogenic bacteria by the host is initially mediated by the innate immune response through recognition of pathogen-associated molecular patterns by the Toll-like receptors [15,16]. Moreover, since the oral cavity as well as other mucosal surfaces, are continuously colonized with non-pathogenic bacteria, there has to be an endogenous negative regulatory mechanism for TLR signaling to prevent an overt host response with deleterious consequences. An example of the consequences of deregulated TLR signaling is Crohn's disease, which is associated with genetic mutations in TLR signaling intermediates [17,18].

Host response to periodontal infection requires expression of a number of bioactive agents, including pro- and anti-inflammatory cytokines, growth factors and enzymes which are the result of the activation of multiple signaling pathways. This activation of intracellular signaling may initiate exclusively as an innate immune response associated with TLR-mediated sensing of PAMPs. However, the biological mediators expressed as a result of TLR-signaling include co-stimulatory molecules involved in the induction of adaptive immunity [16]. This results in a cascade of events that will establish very complex cytokine and signaling networks.

There is abundant evidence indicating that the adaptive immune response, including humoral and cellular aspects, are fundamentally important in mediating the host response to microorganisms of the oral biofilm and also in tissue destruction associated with periodontal diseases [19-24]. Even though cells participating in the adaptive immune response are considered by some authors to be primary source of cytokines leading to bone resorption [25], there is evidence demonstrating that this may occur in the absence of B and T cells [22, 26,27].

Innate immunity and inflammation are not synonymous; however inflammation arises primarily in response to infection. To understand how inflammation is initiated in response to microorganisms it is necessary to focus on the primary interactions between these and the host

cells, which is carried out by the innate immunity. In this sense, TLR signaling is considered the most important interface between the host and the microbes [16]. Considering that these series of reviews focus on host-microbe interactions and based on the fundamental role played by the innate immune system in these events, we chose to emphasize the role of p38 MAPK signaling pathway in the innate immune response in the initiation of periodontal disease. However, the reader should be aware of the crucial role of the adaptive immune response, induced by innate immunity, to periodontal disease progression.

In this complex scenario of host-microbe interactions involving innate and adaptive responses, the signaling pathways originally shown to be relevant for stress, inflammatory and infectious extracellular stimuli are of special interest to therapeutic manipulation. Ideally, these rather 'specialized' pathways that signal stress and inflammatory signals would be selectively modulated to prevent tissue destruction without affecting the host response to prevent dissemination of infection.

In the current paradigm of periodontal disease specific periodontal pathogens are necessary for disease initiation; however, the extent and severity of tissue destruction are largely dependent on the nature of the host-microbial interactions. These interactions are dynamic, since both the microbial composition of the dental biofilm and the competency of host immune responses can vary in the same individual over time. This concept was developed in parallel to the advances on the understanding of the immune response, and research on periodontal disease has been emphasizing mechanisms of host-microbial interactions to understand the disease process, as well as for the development of novel therapeutic strategies. Our research group has been investigating the role of p38 MAPK signaling pathway on host-microbial interactions during periodontal disease. This review intends to discuss the significance of the p38 MAPK pathway and the potential to manipulate this pathway for therapeutic applications *in vivo*.

2. MOLECULAR MECHANISMS OF HOST-MICROBIAL INTERACTIONS IN PERIODONTAL DISEASE

Ever since the initial description of Toll-like receptors (TLRs) in the mid-late 90s [28], the field of innate immunity has been greatly stimulated and the implications of these receptors on the regulation of host response has been intensively studied. Importantly, the roles of TLRs in inflammation and immune response have been expanded, so it is now known that these receptors not only recognize various microbial-associated molecular patterns to activate innate immune response, but they can also bind to endogenous molecules derived from damaged tissue and have a role in inflammation and adaptive immune response. The TLR family currently consists of more than 13 members, each capable of recognizing different PAMPs. These receptors are expressed by immune cells such as macrophages, neutrophils and dendritic cells as well as by non-immune resident cells, such as periodontal fibroblasts [29] and gingival epithelial cells [30]. In periodontal tissues, expression of TLR2 and TLR4 has been positively correlated with inflammation [31,32], as well as in intestinal inflammation [33]. On the other hand, decreased expression of TLR mRNA in the oral mucosa of periodontitis patients has been reported, however concomitantly with increased infiltration of this mucosa with TLR-positive inflammatory cells. This has been regarded by the authors as a possible result of the repeated and prolonged challenge of this tissue with PAMPs and an attempt of the host to reestablish tissue homeostasis, as in an immune tolerance mechanism [34].

TLRs are single-pass transmembrane proteins with an N-terminal presenting leucine-rich repeats that are responsible for the recognition of their ligands and with a C-terminal cytoplasmic domain that is very similar to the cytoplasmic region of the interleukin-1 receptor [35]. Nucleotide-oligomerization domain (Nod) proteins are cytosolic proteins that also have

leucine-rich repeats and were initially described as 'intracellular TLRs' that recognize PAMPs associated with bacteria invading the cytosol [36,37]; however these proteins have also been shown to modulate various signaling pathways, including p38 MAPK and NF- κ B. Our research group has observed that Nod1 and Nod2 are required for transcriptional activation of RANKL mediated by TLR2 and TLR4 signaling; however only Nod1 is needed for expression of RANKL mRNA induced by IL-1 receptor signaling (unpublished data). This illustrates the complexity of TLR signaling and the cross-talk with other signaling pathways involved since the cytosolic domains of TLRs and IL-1 receptor are similar.

Thus, subsequent to recognition of a ligand by TLRs the signal generated utilizes pathways similar to those utilized by the IL-1 receptor; however TLR signaling was originally described in the context of the activation of IRF family of transcription factors and NF- κ B, leading to the expression of interferon- γ and early-response inflammatory genes, respectively. The critical role of TLR receptors in adaptive and immune responses can be used therapeutically to treat infectious diseases, allergies and tumors. Agonists for TLR receptors that enhance innate and adaptive immune responses include ligands of TLR7 (imidazoquinolines) and TLR9 (CpG DNA) that can be used conditions such as basal cell carcinoma, non-Hodgkin's lymphomas, melanoma and allergies [38].

Interestingly, the participation of at least four adaptor proteins containing Toll/IL-1 receptor (TIR) domains that can be recruited by activated TLRs results in important branching of the signal transduction and yields a significant flexibility to TLR signaling by allowing cross-talk with other pathways, including MAP kinase, PKR and Notch pathways (Fig. 1). These adaptor proteins are recruited by TLRs by homophilic interactions between their TIR domains and are utilized differently by the TLRs. TLR5, TLR7 and TLR9 were shown to depend on recruitment of MyD88 to signal [39,40], whereas TLR3 is the only TLR that does not use MyD88 [41]. TLR4, on the other hand, can use all four adaptor proteins: MyD88, TRIF, Mal/TIRAP and TRAM [41-43]. Even though activation of the canonical NF- κ B pathway is usually effected by all TLRs, the timing of NF- κ B activation [44,45] as well as the additional signaling pathways that are activated by the branching of the signal varies among TLR receptors and with the participation of different adaptor proteins. These variations will ultimately affect the biological result in terms of gene expression and can provide opportunities for therapeutic manipulation of signaling by some of the pathways activated by cross-talk. This is demonstrated by the finding that even though NF- κ B activation is observed after TLR4 stimulation by LPS, this may or may not result in inflammatory gene expression depending on the adaptor protein used. In wild-type cells, LPS stimulation results in inflammatory cytokine expression, whereas in MyD88-deficient cells LPS fails to induce cytokine expression. In the absence of MyD88, activation of NF- κ B occurs with delayed kinetics in comparison to wild-type cells [44]. This delayed activation of NF- κ B is dependent on TRIF, and interestingly both pathways involve activation of TRAF6/TAK1 which are common upstream activators of other signaling pathways such as MAP kinases.

The shift on the microbial population present in the oral biofilm from predominantly Gram-positive to Gram-negative bacteria that is associated with the onset of periodontal disease may lead to different patterns of immune response as a result of the type of TLR predominantly activated. Gram-positive bacteria were shown to activate TLR2, which induced increased expression of IL-8, whereas Gram-negative bacteria activated predominantly TLR4, resulting in increased expression of TNF- α [46].

However, some Gram-negative microorganisms that are present in the oral biofilm and associated with periodontal disease are rather unique in their capacity to activate NF- κ B via preferential utilization of TLR2 [47]. Recently, it was reported that most Gram-negative bacteria associated with periodontal disease, including *Porphyromonas gingivalis*, *Tannerella*

forsythisis, *Prevotella intermedia*, *Prevotella nigrescences*, *Fusobacterium nucleatum*, *Aggregatibacter actinomycetemcomitans* and *Veillonella parvula* are all capable of activating TLR2, whereas the latter two microorganisms can also activate TLR4 [48]. Even though all these disease-associated microorganisms activate TLR2 signaling, this pathway can also be activated *in vitro* by microorganisms present in an oral biofilm composed primarily by Gram-positive bacteria, and which are common colonizers of the oral biofilm and not associated with clinical signs of periodontal disease [49]. The fact that TLR2 is activated by both pathogenic and non-pathogenic microorganisms is an interesting finding and suggests differences on the utilization of adaptor proteins and/or concomitant activation of other TLRs by different PAMPs expressed by the various bacterial species that are present in an oral biofilm associated with disease. These differences can lead to the activation of different signaling pathways and subsequent modulation of the host response. It is important to bear in mind the complexity of the oral biofilm, which may include over 500 different microbial species and, consequently, a multitude of PAMPs that can activate various TLRs. The rationale for therapeutic manipulation of signaling pathways that are relevant for expression of genes associated with tissue destruction and disease progression is actually strengthened by this enormous variability of microbial species and PAMPs in the dental biofilm, since an antimicrobial approach is extremely complicated not only by the variability of species but also due to the organization of these microorganisms in a biofilm.

Modulation of TLR signaling by endogenous mechanisms for negative modulation of TLR signaling evolved with the immune system initially in areas of interactions between the host and nonpathogenic microbes [16]. This contact with commensal bacteria through mucosal surfaces is believed to be important during post-natal development, however the local and systemic immune responses are downregulated and reprogrammed by tolerance mechanisms [50,51]. This immune tolerance towards commensal microorganisms combined to adequate responsiveness to pathogens is essential to maintain immune homeostasis while preventing life-threatening infections. Specifically in the oral mucosa, it is not clear how the immune system is able to quickly distinguish between commensal and pathogenic bacteria and tailor the host response [34]. This type of response is observed in intestinal cells which downregulate expression of TLR and adaptor proteins to limit LPS signaling [52], which has also been shown in macrophages [53]. Other mechanisms of tolerance may not involve TLR expression directly, but rather the downstream signaling pathways [54].

This negative regulation can occur by two main mechanisms: 1) cessation of the signal by the clearing/removal of the ligands, and 2) prevention of further signaling. The first mechanism is associated with the resolution of an infection, which results in the removal and clearing of all microbial-associated molecular patterns and, consequently, cessation of TLR signaling. The second mechanism encompasses various endogenous regulatory strategies that interfere with signaling, including receptor expression/degradation, sequestration of adaptor proteins and other signaling intermediates by other proteins that either target these for degradation by the ubiquitin/proteasome or block the kinase activity of the signaling intermediates. These strategies will prevent further downstream signaling and may be somewhat specific for some of the signaling pathways activated downstream of TLR signaling.

Therapeutic manipulation involving inhibition of TLR signaling can be beneficial in autoimmune conditions, such as systemic lupus erythematosus (SLE) that are associated with enhanced production of type I interferon. Other applications of TLR inhibitors include inflammatory diseases and prevention of septic shock. Indeed, a small molecule inhibitor TAK-242 was discovered as a new therapeutic agent for sepsis, and it was shown to function by inhibiting TLR4-specific TRAM-TRIF mediated pathway. Inhibition of this pathway prevents MAP kinase activation and, consequently, pro-inflammatory cytokine production upon stimulation by LPS [55]. In spite of its potential as therapeutic targets to modulate

host-microbial interactions, inhibition of TLR signaling implicates in decreased efficacy of innate immune response with the associated risks to the host in infectious diseases.

3. SIGNALING PATHWAYS IN PERIODONTAL PATHOGEN-INDUCED INFLAMMATION

The hallmark of destructive periodontal disease is the overproduction of cytokines and other inflammatory mediators, which is similar to other chronic inflammatory diseases, including conditions of non-infectious origin such as rheumatoid arthritis [6]. Production of cytokines and inflammatory mediators is usually a tightly-controlled process which is always initiated by external stimuli, or 'signals' that are rapidly transduced through the cytoplasm and into the nucleus where gene expression starts with the transcription of DNA into pre-mRNA. From this very start to the final assembly of the biologically-active protein, there are a great number of regulatory mechanisms that can affect gene expression and various signaling pathways can participate in many of these mechanisms, both at transcriptional and post-transcriptional levels.

The MAP kinases are a group of conserved cytoplasmic kinases that are organized in modules (MAPKKK $\Rightarrow$ MAPKK $\Rightarrow$ MAPK) sequentially activated by dual phosphorylation at Tyrosine/Threonine residues. Of the four distinct classes of MAP kinases described to date in mammals, p38 (α , β , γ and δ isoforms), c-Jun N-terminal activated kinases (JNK1-3) and extracellular activated kinases (ERK1,2) are the most studied. Downstream substrates of MAP kinases include a variety of transcription factors, RNA-binding proteins and other kinases (MAPKAPK - mitogen activated protein kinase-activated protein kinases) that are involved in regulation of gene expression by transcriptional, post-transcriptional, translational and post-translational mechanisms. This implies that therapeutic modulation of signaling pathways can affect various genes, depending not only on the pathway but also on the relative position targeted for inhibition in the signaling cascade.

Interestingly, the proteins comprising many of the signaling pathways are much conserved among different species of organisms indicating their fundamental role in many essential physiological processes. Some of these signaling pathways have also a relevant role in diverse pathological conditions, demonstrating their multivalency. For instance, the p38 MAPK pathway was originally described as critically important to signal stress, inflammatory and infectious stimuli; but it is also involved in the control of fundamental processes including cell proliferation [56], differentiation [57] and migration [58]. Nevertheless, many reports indicate its relevance and/or potential therapeutic application in disease processes that involves inflammation and immunity, including rheumatoid arthritis [59-61], ischemic heart disease [62], allergies [63,64], chronic obstructive pulmonary diseases [65], Alzheimer's disease [66] and cancer [67].

Surprisingly, in spite of evidence indicating a role of p38 MAPK in all these diseases, there is a relative paucity of information regarding its role in oral inflammation-related conditions including temporo-mandibular joint disorders, chronic oral pain and inflammatory changes of the oral mucosa [68]. Interest in its role in chronic inflammatory periodontal diseases has occurred only in the past few years. Our lab group has shown the relevance of p38 MAPK for the regulation of expression of pro-inflammatory cytokines and enzymes induced by inflammatory and infectious signals *in vitro*, including IL-6 [69,70], MMP-13 [29,71] and RANKL [72] in periodontally-relevant resident cells, such as fibroblasts and osteoblasts. This information obtained *in vitro* was also tested in *in vivo* models of periodontal disease and other inflammation-associated diseases, as discussed later in this review (Table 1).

Specifically in periodontal disease, in spite of a great deal of information available on the regulation and expression of inflammatory cytokines, there are only a few reports on the

signaling pathways activated *in vivo*. Nuclear factor-kappaB (NF-kappaB) has been shown to be associated with increased periodontal disease severity [73]. Our research group has found interesting differences on the activation of signaling pathways in two frequently used murine models of experimentally-induced periodontal disease. In both the LPS injection model and the ligature model p38 and ERK MAP kinases, as well as NF-κB was activated, but with different kinetics. On the other hand, activation of JAK-STAT signaling was only observed with the ligature model (unpublished data). The cytokine profile associated with periodontal disease *in vivo* varies and includes both Th1- and Th2-type responses. IL-1α, IL-1β, IL-8 and TNF-α mRNA were detected in macrophages present in inflamed gingival tissues [74], whereas Th-2 cytokine IL-4 and pleiotropic IL-6 protein were also observed in diseased periodontal tissues [75]. A characteristic cytokine profile has been associated with each type of periodontal disease, i.e. inflammation of marginal soft tissues without active bone resorption (gingivitis) or with active bone resorption (periodontitis). Thus, expression of Th1-type cytokines has been associated with gingivitis, whereas Th2 cytokines were found in higher levels on periodontitis-affected tissues [76,77], even though this distinction was not clear-cut with both Th1 and Th2 cytokines being produced in gingivitis- and periodontitis-affected tissues and the predominant profile may actually represent the current activity of tissue destruction [78-80].

The pivotal role of TLR signaling, and that of the innate immune response, in the initiation of periodontal disease is supported by recent findings demonstrating a positive correlation between clinical parameters of gingivitis and periodontitis and TLR4 stimulating ability of supragingival plaque microorganisms [49]. According to current paradigm of periodontal diseases, formation of supragingival plaque is required for initiation of marginal inflammation and subsequent maturation and formation of subgingival plaque [13,81]. Most bacteria from subgingival plaque, on the other hand, have been shown to predominantly stimulate TLR2 with only *A. actinomycetemcomitans* and *V. parvula* stimulating TLR4 [48]. This differential activation of TLR signaling pathways by different bacteria in the oral biofilm can influence the production of cytokines, e.g. stimulation of human whole blood cells with Gram-positive bacteria increased the expression of IL-8, whereas Gram-negative bacteria induced the expression of TNF-α [46]. This may also be relevant in the establishment of a Th1 or Th2 type of host response.

Based on these cytokine profiles, it is expected that p38 MAP kinase shall play a relevant role in disease progression, since this signaling pathway is not only one of the main downstream effectors of TLR signaling [15,16], but is also particularly relevant for the activation and development of adaptive immune responses, as demonstrated by its role on T-cell proliferation and cytokine production [82,83] and differentiation of immature T cells into Th1 or Th2 effector cells [84]. p38 MAPK is also involved in B-cell activation [85] and production of cytokines, including IL-10 [86] and even modulates IL-4-mediated responses in B cells by cross-talk with STAT6 [87]. This illustrates the multiple roles of this signaling pathway and how modulation of its activity may have multiple effects both on innate and adaptive immunity. Other signaling pathways that have been shown to be activated and involved in regulation of gene expression during inflammation and immune response such as Notch [88,89], Wnt [90, 91] and PI3-kinase [92,93] pathways participate in host-microbe interactions, but have not been studied in the context of periodontal disease.

Since the cytokine network established in diseased periodontal tissues is very complex and may be subject to shifts depending on disease activity [94], and also due to the redundant and overlapping role of many cytokines, understanding the signaling pathways involved in cytokine gene expression may provide an alternative approach for the modulation of host response affecting the whole cytokine profile.

4. p38 MAPK—KEY TO INNATE IMMUNE RESPONSE THROUGH POSTTRANSCRIPTIONAL GENE REGULATION

Cells of the immune system keep rigid control over the production of potentially harmful cytokines by repressing their expression at the post-transcriptional level. The adenine and uridine (AU)-rich elements (ARE), located in the 3' untranslated region (UTR) of many cytokines (e.g. GM-CSF, TNF α , IL-2, IL-3, IL-6) and other proinflammatory factors (e.g. COX-2 and MMP-13), plays a major role in post-transcriptional repression. The presence of an ARE in a particular transcript can target it for rapid degradation or inhibit translation.

Inflammatory stimuli (LPS, IL-1 β , or TNF α) dictate mRNA stability through signaling mechanisms. In the presence of inflammatory stimuli, AREs from 3' UTRs of IL-6, IL-8, COX-2, and TNF- α mediate regulation of mRNA stability by p38 MAPK [70,95-100]. p38 MAPK is phosphorylated and activated by upstream kinases MKK3 and MKK6 when stimulated by IL-1 β , TNF- α or LPS [101]. p38 MAPK then phosphorylates MK2 [102] which phosphorylates RNA binding proteins to control mRNA stability [95,97,103].

Ramifications of Blocking/Antagonizing p38 Signaling

Manipulation of signaling pathways is potentially very promising for therapeutic applications in periodontal diseases because it can affect the expression of many cytokines, resulting in a more comprehensive and thorough change in the cytokine network established by the host response to the microbial aggression. Considering the association of p38 MAPK pathway with signaling of stress and inflammatory/infectious stimuli, we have focused on studying the potential of modulating this pathway to affect the expression of some pro-inflammatory cytokines that are especially relevant for host-mediated degradation of mineralized and non-mineralized tissues in periodontal disease.

In vitro evidence for the relevance of p38 MAPK to periodontal disease is primarily derived from studies demonstrating the important role of this signaling pathway to the regulation of expression of inflammatory cytokines that are relevant to the disease process. The cytokines directly or indirectly regulated by p38 MAPK include IL-1 β , IL-4, IL-6, IFN- γ , TNF- α , NO, PGE₂, MMP-13, RANKL in various cell types associated with innate and adaptive immune responses [59,84,104-107]. This role of p38 on regulation of relevant cytokines has been demonstrated also for resident periodontal cells, especially gingival and periodontal ligament fibroblasts [29,69-71,108]. The fact that p38 MAPK regulates the expression of various inflammatory mediators is especially important for therapeutic applications if one considers that targeting expression of a single cytokine may not be effective due to compensation of its biological role by other pro-inflammatory cytokines. However, a significant challenge for this approach is represented by two characteristics of signaling pathways: 1) branching, which allows the establishment of complex signaling networks, because a given signaling intermediate can be activated by different upstream activators, and this same intermediate signaling protein can also activate different downstream effectors; and 2) multivalency, which refers to the diversity of effects a given signaling pathway may have on cell biology, depending on the nature of external stimulation, duration and intensity of stimulation, cell type and differentiation status.

The branching of signaling pathways allows for multiple regulation points along the pathway and can compensate a decrease in activity of other signaling pathways through cross-talk. Thus, depending on the level targeted for modulation in a given signaling pathway, inhibition of a given signaling pathway may have unwanted effects on the activity of other signaling pathways and consequently on the cytokine network. For instance, targeted inhibition of upstream MAP3Ks, such as MEK1, 2 or 3 individually result in completely different patterns of gene

expression in spite of the fact that these kinases are all upstream activators of JNK MAPkinase [109]. However, MEK3 is also an upstream activator of p38 MAPK. We have observed cross-talk between ERK and p38 MAPK signaling pathways in fibroblasts even when targeting p38 MAPK, which is downstream in the signaling pathways [71]. Interestingly, we observed that the p38 MAPK has opposite effects on the regulation of the same gene (MMP-13) depending on the nature of the external stimulation (inflammatory cytokines or bacterial LPS) [29,71]. This type of *in vitro* data suggests that in a situation such as periodontal disease in which multiple external stimuli are present, a network of activated signaling pathways is established and the role of each signaling pathway has to be studied and understood in the context of each cell type and disease model, but it should also be confirmed in *in vivo* models.

The multivalency of signaling pathways also poses a challenge to their therapeutic manipulation because it may not only affect expression of pro-inflammatory cytokines, but also expression of essential genes and bioactive molecules associated with cell proliferation, differentiation and survival. p38 MAPK can be activated by signaling through different receptors, including G-protein-coupled receptors, growth factor receptors, cytokine receptors and Toll-like receptors, which demonstrates the multivalency of this pathway to modulate cell response to a host of extracellular environmental cues by regulation of various genes and cell biology aspects. The fact that p38 is activated by different receptors implicate that various upstream activators are involved in the transduction of the signal, including ASK1, MLK3, MEKK2-4, Tpl2 and TBK1 [110]. These kinases, in turn, are activated by different stimuli in various cell types, and they activate multiple signaling pathways besides p38 MAPK. Targetting these upstream kinases, although still viable for immuno-modulatory purposes, may result in unwanted side-effects because it would also affect other signaling pathways activated downstream. In fact, these negative effects may occur even when modulation of signaling is targeted to occur on downstream mediators of the pathway, such as p38 MAPK itself, either by negative or positive feedback and cross-talk mechanisms.

The difficulties associated with branching and multivalency of p38 MAPK pathway are observed *in vitro*, but may be significantly amplified *in vivo* because of the participation of multiple cell types, which can have different patterns of expression of the upstream activators MAP3Ks or their targets [111]. Various cell types can also utilize the same signaling pathways in a distinct manner due to variability on expression of specific genes, on differential transcription profile, on alternative splicing of signaling proteins and on the pattern of expression of different isoforms of signaling proteins. Notably, even in the same cell type p38 MAPK can have opposite effects on the expression of the same gene, depending on the nature of the external stimulation that induced activation of this pathway. We have shown in fibroblasts that p38 MAPK has a negative regulatory effect on cytokine-induced MMP-13 expression [71], whereas in the same cells p38 had a positive regulatory effect on LPS-induced MMP-13 expression [29]. This antagonistic effect of p38 MAPK by signaling through cytokine and TLR receptors may be associated with differential activation and utilization of upstream activators of p38 MAPK, such as MKK3 and MKK6 and subsequently preferential activation of some isoforms of p38 MAPK by either upstream MAP2K. It also has to be considered that p38 may be involved in different gene regulation mechanisms, including transcriptional and post-transcriptional mechanisms. We have shown that p38 regulates cytokine-induced IL-6 at the level of mRNA stability involving multiple AU-rich elements in the 3'UTR region [70, 112], whereas this signaling pathway regulates cytokine-induced RANKL [72] and LPS-induced MMP-13 [29] by transcriptional mechanisms.

The list of known substrates of p38 MAPK increases frequently and includes many transcription factors (ATF2 [113], ATF1 [114], Sap1 [115], p53 [116], C/EBP β [117], STAT1 [118], Elk-1 [119], Pax6 [120]), other protein kinases (MK2 [121], MK3 [122], MNK1 [123], MSK [124], PRAK [125]) and protein substrates (cPLA2 [126], EGFR [127], Bcl-2 and

Bcl-xL [128], TTP [129,130]). This adds to the complexity of the implications of inhibiting p38 MAPK, which may modulate regulation of gene expression by transcriptional, post-transcriptional and post-translational [131] mechanisms.

Moreover, the recognition of four isoforms of p38 MAPK (α , β , γ and δ) which share only 60% sequence identity with one another suggests that selective activation of these isoforms may occur in specific cell types in response to the combinations of upstream activators. MKK3 and MKK6 were shown to activate p38 $\alpha/\gamma/\delta$, whereas p38 β is preferentially activated by MKK6 [101]. Interestingly, in contrast to α and β isoforms, p38 γ and p38 δ are not sensible to inhibition by pyridinyl imidazole compounds, and there is some evidence for distinct roles for these isoforms. For example, a specific role for p38 δ in human keratinocyte differentiation has been shown [132], and the substrate specificities of the isoform are also different, since p38 α/β are capable of phosphorylating MK2; whereas p38 γ/δ are not. The functional role of p38 γ/δ is still largely unknown, and even though not fully characterized, mice lacking expression of these isoforms are viable, fertile and do not have an obvious phenotype [133].

4. SIGNIFICANCE OF *IN VIVO* BLOCKAGE OF P38 MAPK SIGNALING IN PERIODONTAL DISEASE PROGRESSION

The current concept of periodontal therapy focuses on eliminating bacteria through mechanical means and chemotherapeutics. However, none of these methods has proven universally efficacious, particularly in the case of tissue-invasive species like *A. actinomycetemcomitans* [134]. Thus, the concept of host modulation has garnered much attention in periodontal research over the past decade.

Many host modulatory therapies have been implemented to target the host defenses in periodontal infections. Multiple studies have shown significant clinical improvement and reduction of alveolar bone destruction by modulating arachidonic acid metabolites [135-137] and matrix metalloproteinases (MMPs) [138,139]. Successful attempts have been made to alter osteoclast activity through bisphosphonates [140,141] and a novel vacuolar ATPase [142]. However, these therapies target singular mechanisms of alveolar bone destruction.

One of the attractive features of modulating p38 MAPK signaling is that this molecular target is an 'upstream' common signaling intermediate to many inflammatory cytokines. Activated monocytes, macrophages, and fibroblasts in the periodontium produce cytokines and prostanoids, including TNF- α , IL-1 β , IL-6, and prostaglandin E2 [143]. These cytokines then induce the production of other inflammatory mediators, such as MMPs, prostaglandins, and RANKL that ultimately lead to osteoclastogenesis and tissue destruction [144,145]. Recent evidence reveals that C5a-potentiated IL-6 and TNF- α production by peripheral blood mononuclear cells is inhibited by the p38 inhibitor [146]. Thus, blockade of p38 MAPK could affect inflammation at multiple levels in the immune response.

Several monocytochrome-suppressive therapies have gained Federal Drug Administration approval and are currently available. These include the IL-1 inhibitor anakinra (Kineret®; Amgen) and the TNF- α inhibitors adalimumab (Humira®; Abbott), etanercept (Enbrel®, Wyeth) and infliximab (Remicade®, Centocor). These drugs are intended for the treatment of rheumatoid arthritis, psoriasis, Crohn's disease, ulcerative colitis, and ankylosing spondylitis. To date, none have been approved for the treatment of periodontitis. Despite marked clinical improvements and apparent effectiveness of these drugs, there is still a need for improvement. Thus combination therapy may be more efficacious [147]. This may be because cytokines often act synergistically, as with IL-1 and TNF- α . It has been shown that simultaneous blockage of these cytokines is substantially more effective than blocking only one [148]. Consider the first

human trial in which a single dose of p38 inhibitor decreased TNF- α , IL-1 and IL-6 levels by 90% [149].

However, pan-cytokine blockade does pose potential problems since osteoclastogenesis is required for physiological bone turnover and remodeling. In one study, an orally active p38 inhibitor had a slight anabolic effect as shown by quantitative micro computed tomography [150]. These data suggest that p38 inhibitors have a relatively high suppression of osteoclastogenesis without compensatory shut-off of osteoblastic differentiation. However, it is not believed that osteoclastogenesis is completely eliminated by p38 inhibition. Systemically, a number of hormones and cytokines modulate osteoclastogenesis: parathyroid hormone (PTH), calcitriol, PTH-related protein (PTHrP), PGE₂, IL-1 β , IL-6 and IL-11 [6]. Of these, PTH and PTHrP can still activate osteoclastogenesis independently of p38 signaling [151]. Conceptually, this makes p38 inhibitor strategies appealing as a host modulating agent for treatment of periodontitis as physiological bone turnover (induced by PTH/PTHrP) would occur, but inflammatory bone loss (induced by LPS, IL-1 β , TNF α) would be pharmacologically antagonized.

On another cautionary note, potent cytokine blockade could lead to an immunocompromised host. For example, known side effects of TNF- α inhibitors include reactivation of tuberculosis, infection with opportunistic infections, lymphoma, lupus-like syndrome, injection site reactions, rashes and nephritic syndrome [152,153]. p38 MAPK has several known roles within the immune system. It is required for CD40-induced gene expression and proliferation in B lymphocytes [85,154]. It has also been shown to induce apoptosis of CD8+ T cells and induce T-helper 1 (Th1) differentiation and interferon- γ production by CD4+ T cells [82,155]. Thus, it is possible that suppression of these activities could lead to a depressed immune response.

However, the p38 MAPK isoforms have varying sensitivities to p38 inhibitors. *In vitro* assays using early forms of inhibitors (SB203580 and SB202190) demonstrated that only p38 α and p38 β are blocked; p38 γ and p38 δ remain unaffected [156,157]. Furthermore, the isoforms are variously expressed throughout the body, although they can all be expressed in a tissue given the appropriate stimulus [158]. Isoform α is ubiquitous, β is expressed largely in the brain and heart, δ is found in muscle, and γ is mostly in the lung, kidney, gut, and salivary gland epithelium [159]. While p38 MAPK as a whole is associated with the stress response, each isoform has a specific and different action. For example, α induces apoptosis of while β protects cardiac muscle cells [160]. Therefore, p38 MAPK inhibition does not necessarily block all functions of p38 MAPK.

Because p38 α is the isoform most highly implicated in inflammation, p38 α -selective inhibitors are ideal. SD-282, the inhibitor we used in one of our studies [150] is 14.3-fold more selective for p38 α than for p38 β [161]. This confers strong anti-inflammatory action, including blockage of osteolysis, as demonstrated in rats in both rheumatoid arthritis and periodontitis models [59,162].

FUTURE CONSIDERATIONS

Because p38 α is the isoform most highly implicated in inflammation, p38 α -selective inhibitors are ideal. Currently, p38 MAPK inhibitors are in development by Boehringer Ingelheim (BIRB 796), Glaxo-SmithKline (GSK 681323 and 856553), Pfizer (CP-690550 and PH-797804), Roche (R1503), Scios (SCIO-469) and Vertex (VX-702). Most of these drugs are in the midst of clinical trials. For example, VX-702 has been in phase II trials since 2005, and as of late 2006, the company planned to file an investigational new drug (IND) application [163]. Pfizer has several multi-national centers actively recruiting patients for phase II trials of it PH-797804.

Reported adverse effects of p38 inhibitors include dizziness, gastrointestinal disturbances, and hepatotoxicity (i.e. cytochrome p450 interaction) [164]. Testing in dog models revealed adverse neurological effects with high-dose first-generation VX-745, although no such effects were reported in humans [165]. Subsequent modification resulted in a drug (VX-702) that was incapable of crossing the blood-brain barrier. Fortunately, adverse events seem rare. In a prospective, randomized, double-blind trial, 284 patients reported no difference in side effects between 10, 20, 30, or 60 mg of BIRB 796 given twice daily for 8 weeks versus placebo [166].

As is the case with any new therapeutic, further clinical research with more patients and longer follow-up is needed to determine the safety and efficacy before it can be utilized on a widespread basis. Future pharmacologic efforts may focus on alternative approaches such as targeting other molecules in the p38 MAPK pathway (such as the mitogen-activated protein kinase kinases) or increasing inhibitor selectivity by avoiding ATP binding competition [167].

p38 inhibition is an appealing approach across many aspects of medicine. Although it has been investigated heavily for the treatment of rheumatoid arthritis, it has also been associated with a plethora of disease such as diabetes, cancer, chronic obstructive pulmonary disease (COPD) and even avian influenza [164,168-170]. In the dental field alone, the p38 MAPK pathway is linked to periodontitis, mucositis, chronic ulcerative stomatitis, desquamative gingivitis, pemphigus vulgaris, and temporomandibular joint disorder [171]. As understanding of this pathway grows, so too will its potential applications and the opportunity to improve the lifespan and quality of life for millions of patients (Table 1).

Periodontal disease and rheumatoid arthritis (RA) have remarkably similar inflammatory mediator profiles [172-174]. A variety of immune-associated cell populations are responsible for the pathogenesis of periodontal diseases. Within periodontal lesions, activated monocytes, macrophages, and fibroblasts all produce cytokines such as TNF- α , IL-1 β , PGE₂, and IL-6 and have all been found to be significantly elevated in diseased periodontal sites compared to healthy or inactive sites [143,175,176]. These cytokines orchestrate the cascade of destructive events that occur in the periodontal tissues, and trigger the production of an array of inflammatory enzymes and mediators including matrix metalloproteinases (MMPs), prostaglandins, and osteoclasts, thus resulting in irreversible hard and soft tissue damage [144,177]. Due to the similarity of pathogenesis between periodontitis and RA, p38 inhibitors have the potential to effectively manage periodontal disease progression.

Our data using an experimental rat model of alveolar bone loss clearly indicates that inhibiting p38 MAPK has a protective effect on inflammatory alveolar bone loss (see Fig. 2). Previous data from our laboratory has established that the p38 isoform is clearly required for MMP-13, IL-6 and RANKL expression in periodontally relevant cell types including osteoblasts and periodontal ligament fibroblasts [69-72]. *In vivo*, phosphorylated levels of p38 were extremely high experimental periodontal tissues. Recently, we have been able to demonstrate that phosphorylated levels of p38 are higher in diseased periodontal tissues compared to age-matched healthy control tissues (unpublished data).

In summary, the role of p38 inhibitors to have potential beneficial effects in LPS-induced alveolar bone loss. Although p38 inhibitors should be evaluated in infectious periodontal disease models, these data suggest that use of these agents may be considered as novel host modulatory agents in the treatment and management of human chronic periodontitis.

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Fig. 1.

Fig. 2.

Table 1Summary of *In Vivo* Studies Using p38 MAPK Inhibitors and their Results in Terms of Cytokine Gene Regulation

Disease	Model	Species	Outcome/Result	Ref
Endotoxemia	Infusion of bacterial endotoxin in healthy human volunteers	human	Complete prevention of clinical symptoms (temperature, blood pressure, heart rate). Greater than 90% decrease on serum levels of IL-6, IL-8, TNF- α , blockage of soluble-Tumor necrosis factor-related apoptosis-inducing ligand (sTRAIL).	[179, 180]
Neuropathic pain associated with diabetes	Chemically-induced diabetes	rats	Selective decrease of thermal-induced pain response selectively induced in C-fiber primary nociceptors	[161]
Crohn's disease	Double-blind, controlled randomized clinical trial	human	No clinical benefit in comparison to placebo	[166]
Multiple Myeloma	Spontaneous development	mice	Prevention of osteolytic lesions, decreased vascularity and decreased serum paraprotein	[181]
Chronic Obstructive Pulmonary Disease	Tobacco-smoke induced	mice	Reduction of inflammatory infiltrate, decreased expression of IL-6 and Cox-2	[182]
Rheumatoid arthritis	Collagen-induced	mice	Significant clinical improvement, decreased expression of IL-1 β , IL-6 and Cox-2, reversal of cartilage and bone destruction	[60, 183, 184]
Rheumatoid arthritis	Streptococcal cell wall suspension-induced	rats	Attenuation of clinical signs of arthritis, decreased expression of IL-1 β , IL-6, TNF- α , RANKL, MMP-9 and Cox-2	[59]
Rheumatoid arthritis	Spontaneous development in TNF-transgenic animals	mice	Attenuation of cartilage and	[185]

Disease	Model	Species	Outcome/Result	Ref
			bone degradation, decreased expression of IL-1 β and RANKL, decrease on clinical signs of arthritis	
Periodontal disease	LPS-induced oral bone loss	rats	Prevention of inflammation-associated bone loss, significant decrease on expression of IL-1 β , IL-6 and TNF- α	[162{Rogers, 2007 #666}]

*An online search on National Institutes of Health database (<http://www.clinicaltrials.gov>) using 'p38 MAPK' as search string resulted in 16 clinical trials in October of 2008, including 12 (2 phase I and 10 phase II; 7 active and 5 completed) using p38 MAPK inhibitors. Health conditions studied included: coronary heart diseases, chronic obstructive pulmonary disease, cancer, rheumatoid arthritis and neuropathic pain.