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Jennifer Lynn Krauss  
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**'COMPLEMENTARY' IMMUNE EVASION BY ORAL PATHOGEN  
*PORPHYROMONAS GINGIVALIS***

**Jennifer Lynn Krauss**

**A dissertation submitted to the graduate faculty of the University of Louisville  
School of Medicine in partial fulfillment of the requirement for the degree of**

**Doctor of Philosophy**

**University of Louisville, School of Medicine  
Department of Microbiology and Immunology**

**December 2010**



**'COMPLEMENTARY' IMMUNE EVASION BY ORAL PATHOGEN  
*PORPHYROMONAS GINGIVALIS***

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**November 18, 2010**

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**Don Demuth**

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**Venkatakrishna Jala**



*“ No need to hurry.*

*No need to sparkle.*

*No need to be anybody*

*but oneself.”*

*-Virginia Woolf*

## **DEDICATION**

With admiration and reverence, I'd like to dedicate this work to Henrietta Lacks.

Henrietta, you will always be immortal.

Without your contribution, there would be a huge scientific gap.....

roughly the size of the Milky Way.

## ACKNOWLEDGMENTS

Mom, this is a work of non-fiction. No names have been changed, no characters invented, no events fabricated. I would like to thank you for your boundless patience, suspension of disbelief and willingness to ask questions about my seeming weird “scientific world”, all the while secretly fretting that you’d lost your daughter to fiendish blood letters.

I would like to send out many hugs and kisses to the ‘Blair Boys’, my four nephews, who keep trying to convince me that I’m in desperate need of a stunt double at work. This would facilitate the time required to fend off evil storm troopers, erect elaborate Lego® structures and god-willing, become the fifth teenage-mutant ninja turtle.

I have no desire to get mushy or sentimental about extending my gratitude towards my mentor; however, some things are just beyond your control. I can’t thank you enough, Dr. George Hajishengallis, for guiding me through this process with unwavering dedication and professional integrity. You always cheer for the underdog, and I, was no exception. Despite my speckled scientific past and broken spirit, you took me under your wing and chiseled me into worthy academic contender. I hope I will always make you proud.

I could extend a generic acknowledgement to my committee members, but toss in the night I won’t. I am deeply honored that Pascale Alard and Jill Suttles accepted the

invitation to be on my committee....for the second time. Both women have held their own in this male-dominated academic arena and paved the way for other female investigators through their mentorship. What's your secret? Indeed, I was also like to extend a Y-chromosome friendly acknowledgement and show appreciation for the contributions made to my project by Don Demuth and Venkatakrishna Jala.

To the power team of the Hajishengallis lab, I will be forever indebted to you for your humble graciousness. Meg, you've rescued me so many times from the conspiring and often, unforgiving universe. From temperamental refrigerators to collapsing roof gutters to flaky and unreliable lawnmowers, you always came to my aid. You even secured my smashed trunk with cables after my car was regrettably pulverized by a lady with shady insurance who thought there would be no harm in running that red light. Ravi, you are truly one of the most sincere and kindest human beings I've ever known. I'd like to thank you for fronting the ransom money when my car was being held hostage by the City of Louisville. Shuang, thanks for always being the voice of reason and calm logic. Last but certainly not least, I'd like to thank Fenge Li for taking such loving and nurturing care of my experimental mice.

## ABSTRACT

### **'COMPLEMENTARY' IMMUNE EVASION BY ORAL PATHOGEN *PORPHYROMONAS GINGIVALIS***

**Jennifer Lynn Krauss**

**November 18, 2010**

Complement, an early recognition system of innate immunity that senses local tissue damage and infection, cross-talks with and regulates other signaling systems, including Toll-like receptor (TLR) pathways. In the context of periodontitis, destructive inflammation and disease promotion are associated with extensive and synergistic activation of TLRs and complement within the chronically inflamed periodontium. The virulence of the periodontal pathogen *Porphyromonas gingivalis* is dependent, at least in part, upon its ability to use sophisticated stealth and sabotage tactics to undermine innate immunity. Intriguingly, although this pathogen can modulate TLR2 signaling and suppress specific aspects of complement activation (126), it proactively generates an active complement fragment (C5a) through limited degradation of the fifth complement component (C5) by virtue of its C5 convertase-like activity. We hypothesized that this seemingly counterproductive action may provide a survival advantage; permitting *P. gingivalis* to instigate a subversive crosstalk between TLR2 and C5aR. Our work supports this hypothesis by demonstrating that C5a exposure promoted a synergistic rise of intracellular cAMP and impaired the ability of macrophages to destroy *P. gingivalis*. The cAMP synergy strictly required TLR2 signaling and a pertussis toxin- and

thapsigargin-sensitive C5a receptor pathway, whereas protein kinase A and glycogen synthase kinase-3 $\beta$  acted as downstream effectors. Antagonistic blockade of the C5a receptor abrogated this evasive strategy and may thus have important therapeutic implications in treating periodontal disease. This first demonstration of complement-TLR crosstalk for immunosuppressive cAMP signaling indicates that pathogens may not simply undermine complement and/or TLRs as separate entities, but may also exploit their crosstalk pathways.

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## CHAPTER ONE: INTRODUCTION

### **Innate immunity: its role in periodontal disease**

Innate immunity is a phylogenetically ancient system of host defense and represents the inherited resistance to infection (70). Until relatively recently, the innate immune response was viewed as a non-specific and temporary expedient to “buy time” until the activation of adaptive immunity, which comprises the system of B and T lymphocytes, each of which expresses antigen receptors of exquisite specificity (34). Although lacking the ability to make such fine structural distinctions, innate immunity is nevertheless endowed with considerable specificity. Indeed, germ-line encoded receptors (collectively known as pattern-recognition receptors) can detect and respond to conserved and generally distinct microbial structures, which are shared by related groups of microorganisms (*e.g.*, lipopolysaccharide of gram-negative bacteria or lipoteichoic acid of gram-positive bacteria) (104). Most importantly, innate immunity is sophisticated enough to make judgments that instruct the initiation and progression of the adaptive immune response (34) (104). In this regard, the acquired specificity of the antigen receptors is not the result of co-evolution with microbes but the outcome of randomly generated gene recombination. Thus, even though the adaptive immune receptors can bind virtually any structure, they have no clue on the biological context of the encountered antigen (*i.e.*, should they respond or not?). This information, however, is

provided by innate immune mechanisms, which act as mediators between detection of infection and induction of the adaptive response. Not surprisingly, successful pathogens which disarm or subvert host defenses target preferentially innate immunity (36) and particularly central systems such as the complement and the Toll-like receptor (TLR) family of pattern-recognition receptors (88) (137).

In the oral cavity, innate immunity contributes significantly to antimicrobial defense, although inadequate or overexuberant activation of the innate response may lead to oral disease, such as periodontitis (32) (37). In this context, periodontal health represents a dynamic state where pro-inflammatory and antimicrobial activities to control infection are optimally balanced by anti-inflammatory mechanisms to prevent unwarranted inflammation (37). This homeostatic balance may be disrupted, however, either by genetic defects in host immunity or by pathogens that undermine host defense mechanisms (37) (79) (83). It should be noted that pathogen-instigated immune suppression of specific pathways and destructive inflammatory responses in the periodontium are not necessarily mutually exclusive, since the latter may arise as a consequence of the inability to control infection (49).

The ability of periodontal pathogens to persist and establish chronic infections suggests that they may have evolved ways to disarm these defense mechanisms or subvert them to their advantage. Understanding the mechanisms of periodontal host-pathogen interplay can offer important insights into the disease pathogenesis and facilitate the rational design of therapeutic interventions.

## **Complement and TLRs: a potential for crosstalk**

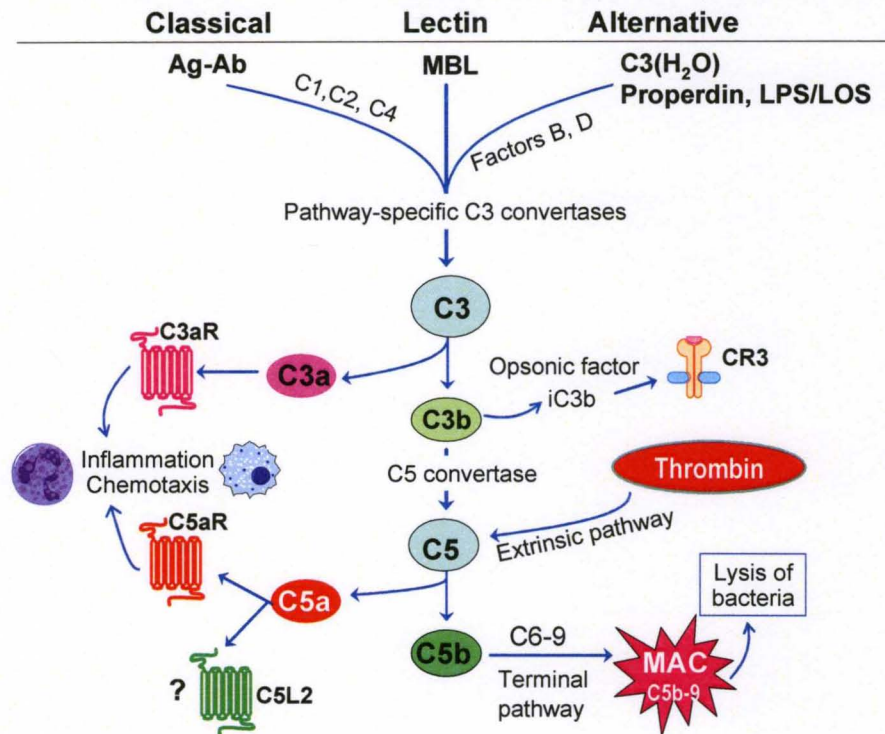
The term "complement" was coined by Paul Ehrlich in the late 1890s to describe a heat-sensitive activity in serum that is *complementary* to that of antibody in causing lysis of bacteria (142). In line with this early view, the complement system has been traditionally considered as an antimicrobial enzyme system found in serum and inflammatory exudates like the gingival crevicular fluid (4) (109) (122). However, it is now well appreciated that complement constitutes a fundamental component of innate immunity, by virtue of its ability to orchestrate critical events during immune and inflammatory responses, including regulation of other innate or adaptive immune pathways (63) (90) (99) (165).

The triggering of the complement system involves sequential activation and proteolytic cleavage of a series of serum proteins, leading to recruitment and activation of inflammatory cells, microbial opsonization and phagocytosis, and direct lysis of targeted pathogens (99). In addition to the serum components, the integrated complement system also includes membrane-bound regulators and receptors for interactions with various mediators of the immune system. Complement activation can proceed through three distinct mechanisms, namely the classical, lectin, or alternative pathways (99) (Fig. 1). All three pathways converge at a central step, involving activation of the third component of complement (C3) by pathway-specific C3 convertases (87) (99). Activation of the classical pathway is initiated by antigen-antibody complexes, whereas the lectin pathway is triggered through interaction of a secreted pattern-recognition receptor (the mannose-

binding lectin) with specific carbohydrate groups found on the surface of a variety of microorganisms. To ensure fast and immediate response to invading pathogens, the complement cascade is maintained at a low level of activity (“tick-over”) by the so-called alternative pathway. This pathway is initiated by spontaneous hydrolysis of C3 to C3(H<sub>2</sub>O), thereby inducing a conformational change that allows binding to complement factor B and formation of the initial alternative pathway C3 convertase. This results in rapid turn-over of the alternative pathway, as long as there is no sufficient negative regulation as normally occurs with non-self surfaces (*e.g.*, bacteria). In addition to this mechanism, the alternative pathway can be induced by bacterial lipopolysaccharide and lipooligosaccharide molecules in a way that strictly requires the participation of the plasma protein properdin (77). The alternative pathway may represent up to 80% of complement activation (99). In all three pathways, proteolytic cleavage of a series of proteins downstream of C3 leads to the generation of effector molecules, including opsonins (C3b, iC3b) and anaphylatoxins (C3a, C5a). The iC3b fragment is generated by further cleavage of microbe-attached C3b and mediates phagocytosis by complement receptor-3 (Fig. 1). The inflammatory anaphylatoxins C3a and C5a activate seven-transmembrane domain G-protein-coupled receptors, known as the C3a receptor and C5a receptor (CD88), respectively. A newly identified but modestly characterized alternative receptor for C5a is the so-called C5a receptor-like 2 (C5L2). Originally believed to be an anti-inflammatory decoy receptor, C5L2 is now thought to play a novel and distinct role in sepsis (160). Another C5 cleavage product, the C5b, initiates the assembly of the C5b-9 membrane attack complex, which induces lysis of complement-targeted bacteria (99) (Fig. 1).

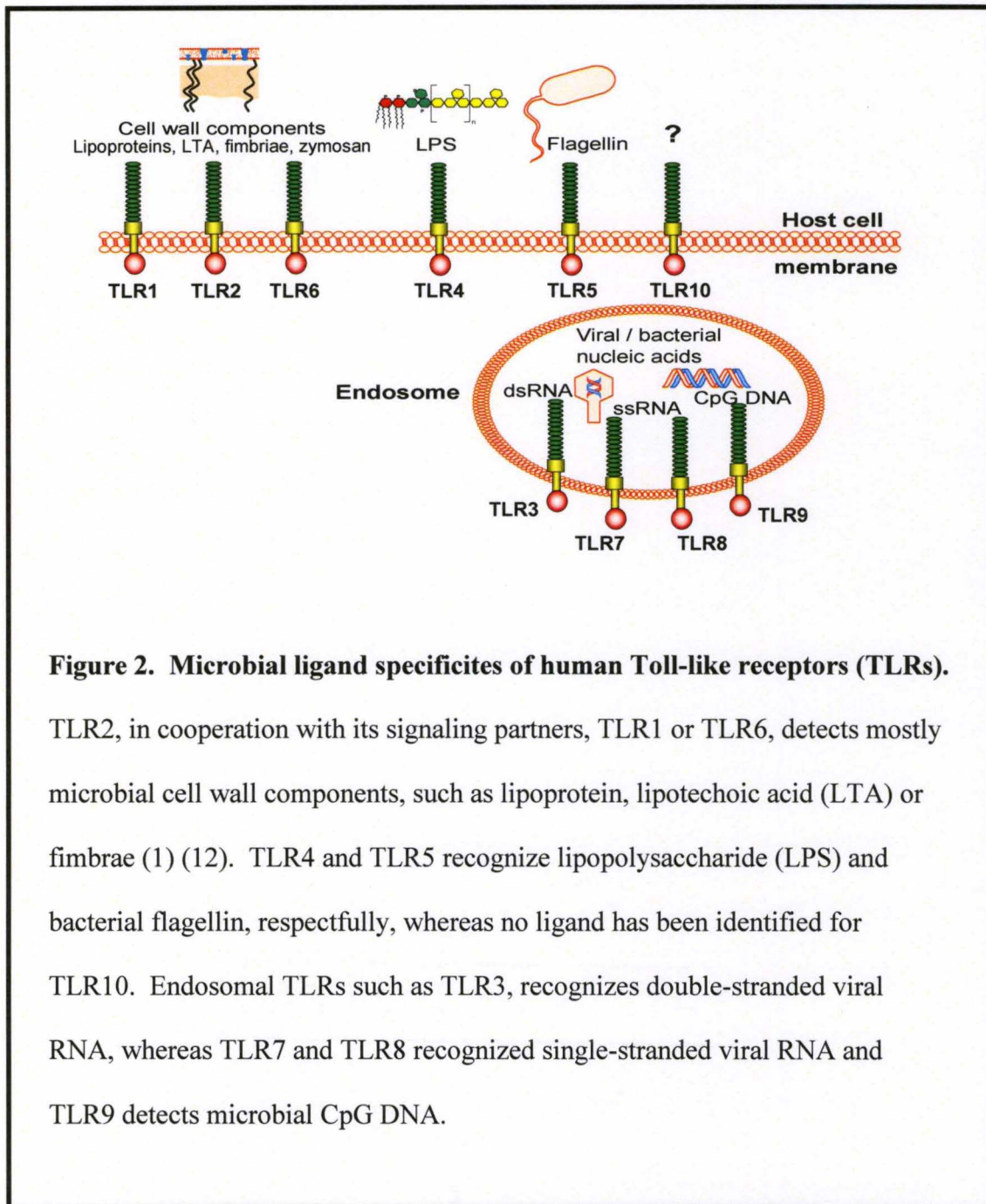
TLRs comprise a family of pattern recognition receptors named after their similarity to the *Drosophila* Toll protein (92) (104). Their discovery in the late 1990s has sparked a resurgent interest in innate immunity. Indeed, the study of TLRs has helped appreciate the economical specificity of the innate immune system and that adaptive immunity did not evolve to replace innate immunity, but rather evolved around it. TLRs are transmembrane glycoproteins comprising an N-terminal leucine-rich repeat domain, a transmembrane region, and a C-terminal cytoplasmic signaling domain (71) (75). These receptors are primarily expressed by first-line professional phagocytes (*e.g.*, neutrophils, macrophages, and dendritic cells) and are thus strategically located for early recognition of microbial pathogens (1). To date, 10 human TLRs have been identified which generally sense and respond to distinct types of microbial structures (Fig. 2). For instance, TLR3 responds to double-stranded viral RNA, TLR4 responds to enterobacterial lipopolysaccharide, TLR5 to bacterial flagellin, and TLR9 to microbial CpG DNA. TLR2 is unique in that it heterodimerizes with signaling partners (TLR1 or TLR6) for detecting and responding to microbial cell wall components, such as lipoteichoic acid, lipoproteins, yeast zymosan or fimbriae (1) (10) (80).





**Figure 1. Activation pathways of the complement system.** All three pathways converge at a central step, involving activation of the third component of complement (C3) by pathway-specific C3 convertases. Proteolytic cleavage of a series of proteins downstream of C3 leads to the generation of potent effector molecules. These include the anaphylatoxins C3a and C5a, which activate specific receptors (C3aR and C5aR). Moreover, C5a also interacts with the modestly characterized C5a receptor-like 2 (C5L2) (81). Additional effectors generated downstream of C3 are the opsonins C3b and iC3b, the latter of which coats microbes and promotes their phagocytosis by complement receptor-3 (CR3). In the terminal pathway, C5b initiates the assembly of the C5b–9 membrane attack complex (MAC), which in turn induces microbial cell lysis (96).

Those TLRs which are mainly responsible for detecting extracellular microbial structures are expressed on the host cell surface (TLRs 1, 2, 4, 5, and 6), whereas those specializing in detecting viral or bacterial nucleic acids are appropriately located intracellularly on endocytic vesicles or organelles (TLRs 3, 7, 8 and 9) (Fig. 2). Following ligand binding, TLR signaling is triggered upon recruitment of adaptor proteins to the cytoplasmic TLR domains, which help propagate the signals to downstream kinases and transcription factors. This ultimately leads to induction of immunoregulatory genes that activate or suppress the innate immune and inflammatory response (116) (117). The presence of both common and selective adaptors, in conjunction with the apparent compartmentalization of the TLRs, allows the induction of individual signaling pathways (for at least some TLRs) in addition to a core TLR response (117). It is thus possible that activation of diverse TLR intracellular pathways, dependent upon different TLR ligand specificities, may allow the host to tailor a response that is appropriate against a given pathogen.



**Figure 2. Microbial ligand specificities of human Toll-like receptors (TLRs).**

TLR2, in cooperation with its signaling partners, TLR1 or TLR6, detects mostly microbial cell wall components, such as lipoprotein, lipoteichoic acid (LTA) or fimbriae (1) (12). TLR4 and TLR5 recognize lipopolysaccharide (LPS) and bacterial flagellin, respectively, whereas no ligand has been identified for TLR10. Endosomal TLRs such as TLR3, recognizes double-stranded viral RNA, whereas TLR7 and TLR8 recognized single-stranded viral RNA and TLR9 detects microbial CpG DNA.

Both complement and TLRs are rapidly activated by most pathogens upon encounter with the host, and common microbial molecules like gram-negative bacterial lipopolysaccharide and yeast zymosan can act both as TLR ligands and complement activators. It is conceivable that the coordination of the early innate response would require a crosstalk between the complement and the TLR systems. In this regard, a systematic analysis of crosstalk in intracellular signaling pathways has revealed that a great number of microbe-induced stimuli converge on a relatively limited number of effector signaling pathways (113). In principle, a molecular crosstalk between complement and TLRs could result in cross-regulation of the two systems, including potential synergistic or even antagonistic interactions. These interactions may help enhance host defense or regulate it to prevent excessive inflammatory responses. However, it is also plausible that at least some crosstalk interactions may be instigated by the pathogens themselves for deregulating or modifying the host response in a way that favors their survival. Though only recently has this issue started to be addressed, available evidence indicates bidirectional cooperation between the complement and the TLR system, since complement regulates TLR activation (63) (165), whereas TLR signaling transmodulates the activity of complement receptors (57) (61).

## **Periodontitis, associated bacteria, and complement/TLR immunity**

Periodontal disease is possibly the most common chronic disorder of infectious origin in humans, resulting in inflammatory destruction of the tooth-supporting tissues (123). The disease is initiated by certain species of subgingival gram-negative anaerobic bacteria co-existing within dynamic communities of highly-organized architecture (24) (144), originally termed “dental plaque” which predates the more modern term “biofilm” (41) (106). In periodontal health, the ordered structure of the dental plaque biofilm consists predominately of gram-positive, facultative anaerobic bacteria, although the onset of the disease is associated with a shift to gram-negative anaerobic bacteria which begin to colonize the subgingival pocket with greater frequencies (145). Using a color-coded system, Socransky and colleagues characterized these microbial communities as red, orange, green, purple and yellow complexes, on the basis of cluster analysis, community ordination, and associated disease severity (146). A high prevalence of red complex members such as *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia* correlates strongly with periodontal tissue destruction (65) (146). *Prevotella intermedia* and *Fusobacterium nucleatum*, both members of the orange complex, are also associated with various forms of periodontal disease (21) (146) (162).

While the bacteria constitute an essential etiologic factor, it is the host inflammatory reaction to bacterial challenge that primarily mediates periodontal tissue damage (37). This is not to say, however, that the challenge in periodontitis involves simply the issue of controlling the inflammatory response. In a related context, purely anti-inflammatory therapies in sepsis clinical trials have generally failed even if the initial hyper-inflammatory stage was controlled; indeed, many patients would succumb to the

infection itself at later stages of the disease (134). Therefore, periodontal and other infection-driven inflammatory diseases should be dealt with in ways that address both infection and inflammation. This in turn requires adequate understanding of both protective and destructive aspects of the host response and how pathogens may evade the former and contribute to the latter.

There is strong evidence that complement and TLRs form an important link between infection and various local or systemic autoimmune or inflammatory conditions, such as septic shock, ulcerative colitis, rheumatoid arthritis, systemic lupus erythematosus, atherosclerosis, ischemia/reperfusion injury, and asthma (2) (17) (131) (160). There is also evidence for complement and TLR involvement in periodontal disease. In this regard, a profusion of complement proteins and derived split products are found within the gingival crevicular fluid of periodontitis patients, composing up to 70% of that found in the serum (127). The functionality of the complement components of the gingival crevicular fluid has been confirmed (18), whereas activated complement fragments have also been detected in the gingival connective tissue (18). Importantly, induction of experimental gingivitis in human volunteers causes progressive elevation of complement cleavage products and correlates with increased microbial plaque accumulation, clinical inflammation, and bleeding on probing (122). These clinical findings suggest a role for complement involvement in periodontal pathogenesis. Moreover, *in vitro* mechanistic studies have demonstrated complex interactions between periodontal bacteria and the complement system (58) (103) (125).

In addition to elevated complement activity, the inflamed periodontium is infiltrated by TLR-expressing inflammatory cells, whereas healthy gingiva display

significantly lower levels of TLR expression (110) (111) (129). Besides professional inflammatory cells, gingival epithelial cells and fibroblasts also express TLRs and the level of expression correlates with disease activity (84) (129) (148) (159). In terms of function, TLRs (particularly TLR2 and to a much lesser extent TLR4) have been shown to regulate important immune and inflammatory responses to periodontal bacteria *in vivo* and *in vitro* (3) (11) (16) (23) (33) (54) (59) (118) (164).

However, the precise roles, whether protective or destructive, played by the complement and the TLRs in periodontal infection and inflammation are poorly understood. This is partly because these issues have not previously been systematically investigated. Nevertheless, a substantial body of available literature exists, which, if properly synthesized and interpreted, could provide important new insights for future studies.

### ***Porphyromonas gingivalis*: master of subversion**

In principle, a host inflammatory response can become destructive when it is deregulated and its magnitude gets out of proportion to the microbial threat, or when it is undermined by pathogens leading to persisting but ineffective inflammation in terms of infection control (37) (49) (83) (135). In the context of periodontitis, *P. gingivalis* could be reasonably characterized as a master of subversion, on the basis of sophisticated sabotage tactics presented below. This gram-negative anaerobic organism expresses an elaborate system of adhesins and proteolytic enzymes (*e.g.*, long and short fimbriae, hemagglutinins, and Arg- and Lys-specific cysteine proteinases known as gingipains), which coordinately enable the pathogen to colonize host tissues and secure critical nutrients (89). As important as these virulence features may be, *P. gingivalis* would

probably be unable to establish a chronic infection, unless it could have also evolved ways to evade, undermine, or trick the host immune system. This is lucidly exemplified by its capacity to not only subvert both complement and TLR immunity but, moreover, to exploit crosstalk signaling pathways between complement and TLRs.

### **Neutralization of complement action**

*P. gingivalis* causes significant inhibition of complement activation, regardless of the initiation pathway involved (classical, lectin, or alternative; Fig. 1), through gingipain-dependent degradation of key complement components, such as the C3 (126) (142). As a consequence, the deposition of opsonins or the membrane attack complex on the pathogen surface is suppressed, unless its gingipain activity is ablated by chemical or genetic means (139) (143). All three gingipain enzymes participate in complement inactivation, although the Arg-specific enzymes (HRgpA and RgpB) are more potent in this regard than the Lys-specific gingipain (Kgp) (125). As a further safety measure, the pathogen appears to hijack physiological mechanisms of inhibiting the complement cascade. In this regard, *P. gingivalis* uses its HRgpA to capture the circulating C4b-binding protein on the bacterial cell surface, thereby acquiring the ability to negatively regulate the classical pathway C3 convertase (128).

The above summarized findings are consistent with observations that *P. gingivalis* is exquisitely resistant to the lytic action of complement (125) (143). Curiously, however, Arg- and Lys-gingipain mutants are as resistant as the wild-type organism upon their exposure to human serum, even though active complement fragments are readily deposited on their bacterial surface (143). These intriguing observations suggest an inherent protective mechanism that is independent of complement inactivation. Indeed, a



surface anionic polysaccharide was implicated in this inherent resistance since *P. gingivalis* mutants lacking this structure become readily susceptible to complement-mediated lysis (143). Although this anionic polysaccharide may directly confer resistance, the possibility for an indirect effect may not formally be ruled out. In this context, certain pathogens (*e.g.*, *Helicobacter pylori* and *Escherichia coli*) acquire resistance against complement lysis by expressing molecules that can bind CD59, a host regulatory protein which inhibits the terminal step of the membrane attack complex formation (88).

It, therefore, appears that *P. gingivalis* may be using a number of different reinforcing mechanisms to ensure its survival in the presence of complement. In this regard, since the inhibitory mechanisms of *P. gingivalis* against complement activation are leaky (125), it makes sense that it has also developed inherent resistance against complement-dependent lysis. However, if the surface anionic polysaccharide is sufficient to provide inherent protection, a plausible question is why the pathogen has additionally evolved ways to suppress a system that cannot kill it. An interesting interpretation is that *P. gingivalis* may have evolved complement inactivation capacity not for its own protection, but for the benefit of other organisms occupying the same subgingival niche. This action may not be as altruistic as it seems; it may actually offer a survival advantage for *P. gingivalis*, as it depends on other periodontal bacteria for enhanced colonization and full expression of virulence (74) (78) (124). Since *P. gingivalis* is resistant to the lytic action of complement (125) (143), the ability of the complement system to directly offer host protection against this organism is seriously questioned. Nevertheless, it cannot be ruled out that complement activation may indirectly fight this pathogen through

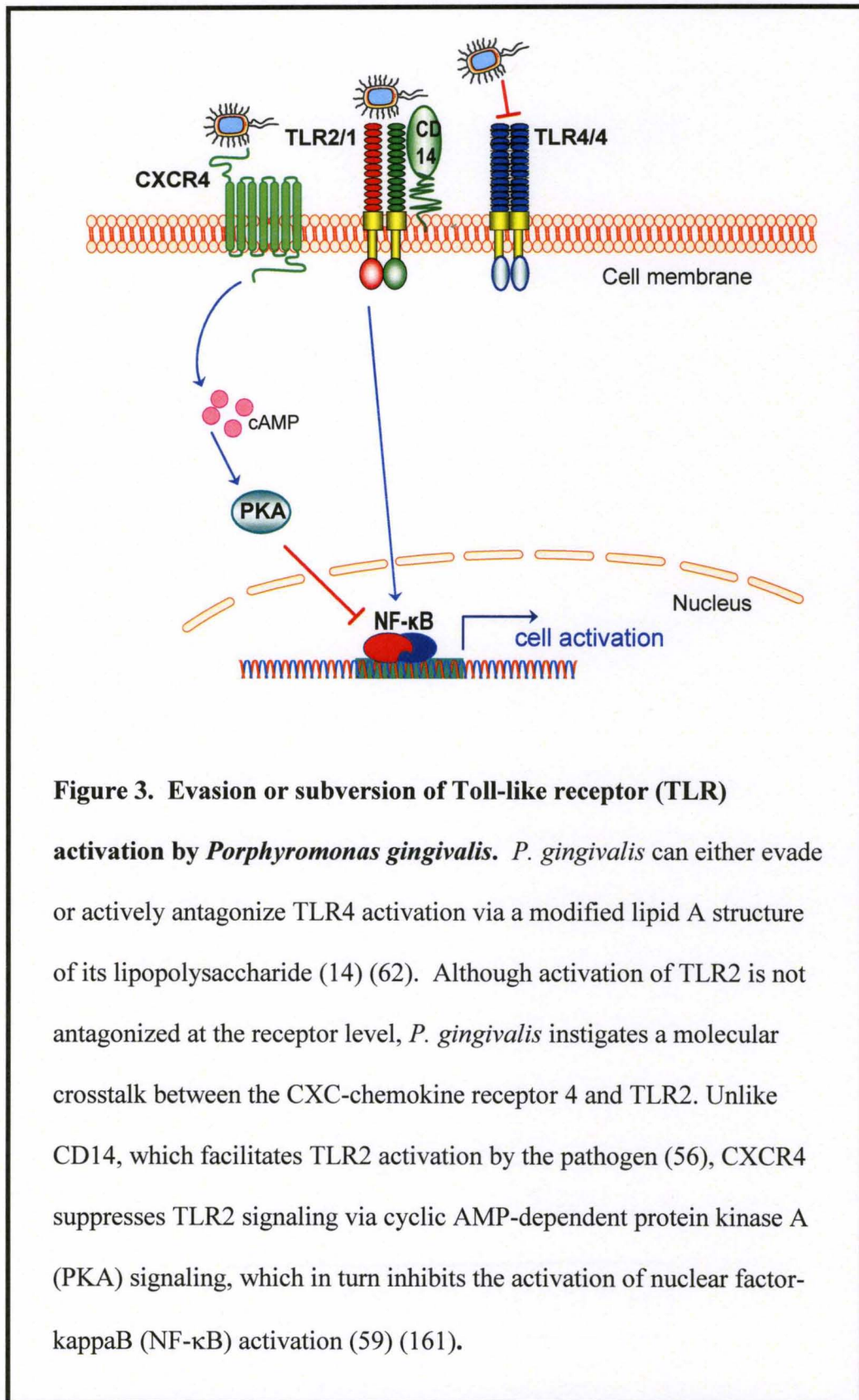
the recruitment and activation of phagocytic cells. However, *P. gingivalis* may have evolved strategies to diminish or evade its destruction by phagocytes in the presence of complement.

### **Evasion and subversion of TLRs**

Available evidence suggests that *P. gingivalis* may have also evolved ways to evade or subvert the TLR system, which senses this organism primarily through TLR2, as shown *in vitro* and *in vivo* (11) (54). On the other hand, TLR4 appears to play little or no role in cell activation in response to this oral bacterium (11) (54). These observations appear curious given that *P. gingivalis* is a gram-negative organism which expresses a lipopolysaccharide. However, the organism elegantly utilizes specific lipid A 1- and 4'-phosphatases and a deacylase which in concert generate a tetra-acylated and dephosphorylated lipid A structure (14). This modification renders the lipopolysaccharide molecule biologically inert, thereby allowing *P. gingivalis* to evade TLR4 activation (14). At the same time, this modification confers protection against polymyxin B and perhaps other cationic anti-microbial peptides (14). Intriguingly, the presence of high concentrations of hemin (an environmental nutrient found in diseased sites) suppresses lipid A 1-phosphatase activity and leads to the production of a mono-phosphorylated lipid A, which actively antagonizes TLR4 activation (14) (15). Thus, even though *P. gingivalis* may express other molecules with intrinsic TLR4 agonistic activity, TLR4 activation is likely suppressed in the context of the whole organism (Fig. 3), as seen both *in vitro* and *in vivo* (11) (54). In this regard, *P. gingivalis* behaves like certain other, non-oral pathogens which have also opted to modify their surface structures so as to escape TLR4 recognition (5) (108) (141).

The above considerations may explain why TLR2, rather than TLR4, is the predominant TLR involved in *P. gingivalis* recognition. Induction of TLR2 signaling by *P. gingivalis* requires a signaling partner (TLR1 or TLR6), takes place in membrane lipid rafts where the receptors are recruited ad hoc, and is facilitated by a non-signaling co-receptor (CD14) which constitutively resides in lipid rafts (54). Although the host TLR2 response may be potentially protective, *P. gingivalis* has developed ways to undermine the intended host response. Indeed, the pathogen was shown to manipulate the TLR2 response by instigating a molecular crosstalk between TLR2 and the CXC-chemokine receptor 4 in macrophage lipid rafts (59). Specifically, the binding of *P. gingivalis* fimbriae to CXC-chemokine receptor 4 induces cAMP-dependent protein kinase A signaling, which in turn suppresses TLR2-dependent activation of nuclear factor- $\kappa$ B and induction of nitric oxide (Fig. 3) (59). The inhibition of production of this key antimicrobial molecule promotes the ability of *P. gingivalis* to survive *in vitro* and *in vivo* (59).

The impact of TLR2 signaling on the ability of *P. gingivalis* to cause experimental periodontitis was examined by two independent studies, which found that TLR2-deficient mice (but not TLR4-deficient or wild-type controls) are protected against periodontal bone loss (11) (42). These findings are consistent with the notion that TLR2 signaling is manipulated by *P. gingivalis* in a way that promotes its virulence. However, an alternative or additional interpretation is that the observed enhanced bone loss in normal mice could be attributed to *P. gingivalis* induction of TLR2-mediated inflammatory osteoclastogenesis (154).



**Figure 3. Evasion or subversion of Toll-like receptor (TLR)**

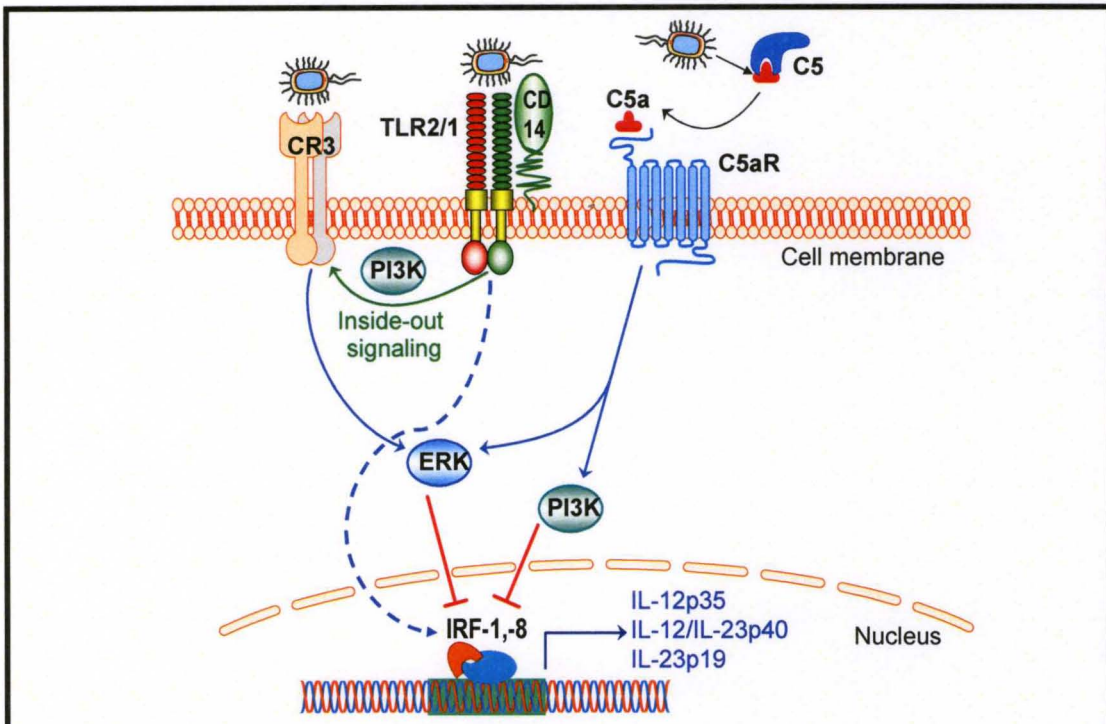
**activation by *Porphyromonas gingivalis*.** *P. gingivalis* can either evade or actively antagonize TLR4 activation via a modified lipid A structure of its lipopolysaccharide (14) (62). Although activation of TLR2 is not antagonized at the receptor level, *P. gingivalis* instigates a molecular crosstalk between the CXC-chemokine receptor 4 and TLR2. Unlike CD14, which facilitates TLR2 activation by the pathogen (56), CXCR4 suppresses TLR2 signaling via cyclic AMP-dependent protein kinase A (PKA) signaling, which in turn inhibits the activation of nuclear factor-kappaB (NF-κB) activation (59) (161).

## Exploitation of crosstalk interactions between TLRs and complement

TLR2 activation by *P. gingivalis* induces two distinct signaling cascades (57). One of the cascades leads to induction of pro-inflammatory and antimicrobial responses, and represents the pathway that is manipulated by *P. gingivalis* through exploitation of CXC-chemokine receptor 4. The other cascade represents a pro-adhesive pathway and involves a crosstalk between TLR2 and the complement system (57). Specifically, *P. gingivalis* induces TLR2 inside-out signaling which transactivates the adhesive capacity of complement receptor-3 (62) (Fig. 4). This crosstalk is made possible by the property of complement receptor-3 to cluster with TLRs in lipid rafts of *P. gingivalis*-stimulated cells (54). Once transactivated, however, complement receptor-3 becomes a target of subversive activity by *P. gingivalis*.

Indeed, *P. gingivalis* uses its fimbriae to bind complement receptor-3, which in turn mediates the uptake of this oral pathogen by macrophages (56). Intriguingly, this phagocytic mechanism does not promote the killing of *P. gingivalis* (158), possibly because complement receptor-3 is not linked to vigorous microbicidal mechanisms (135). In contrast, when *P. gingivalis* is phagocytosed by alternative receptors, *i.e.*, when complement receptor-3 is blocked or genetically ablated, the intracellular killing of this pathogen is dramatically enhanced (158).

The interaction of *P. gingivalis* with complement receptor-3 also activates the extracellular signal-regulated kinase 1/2, which in turn selectively inhibits mRNA expression of the p35 and p40 subunits of interleukin-12 (53) (Fig. 4).



**Figure 4. Crosstalk pathways between Toll-like receptors and complement**

**in *P. gingivalis* activated macrophages.** TLR2 recognition of *P. gingivalis*, induces PI3K-dependent inside-out signaling which transactivates CR3, thereby inducing ERK1/2 signaling that downregulates the expression of messenger RNA for cytokines of the IL-12 family (53) (54). Moreover, *P. gingivalis* uses its gingipains to attack C5, consequently releasing biologically active C5a that can activate PI3K and ERK1/2 through its receptor (C5aR), in turn suppressing critical transcription factors required for expression of cytokines of the IL-12 family (15) (63) (124). Intriguingly, IL-12 inhibition through these mechanisms results in impaired immune clearance of *P. gingivalis* *in vivo* (53), suggesting that the pathogen exploits TLR- complement crosstalk signaling to promote its virulence.

Interleukin-12 (IL-12) is a key cytokine involved in pathogen clearance through regulatory effects on the production of interferon- $\gamma$ , which is a potent activator of the macrophage microbicidal capacity (153). Consistent with the above, wild-type mice elicit lower levels of interleukin-12 and interferon- $\gamma$  and display impaired clearance of *P. gingivalis* systemic infection compared to mice that lack complement receptor-3 (53). Similar results are seen after CR3 blockade with a specific antagonist that suppresses *P. gingivalis* induction of periodontal bone loss in mice (53). In brief, there is compelling evidence that complement receptor-3 constitutes an Achilles' heel which confers host susceptibility to *P. gingivalis* infection. In this regard, it seems likely that *P. gingivalis* may have actually co-opted a natural anti-inflammatory mechanism to evade innate immunity. Specifically, complement receptor-3 is heavily committed to phagocytosis of iC3b-coated apoptotic cells, which are not normally recognized as danger (76) (105). This precludes induction of a vigorous host response and, in fact, production of interleukin-12 is inhibited following phagocytosis of apoptotic cells by macrophages (76).

Although *P. gingivalis* inhibits the complement cascade, curiously enough, the pathogen proactively generates one of the active complement fragments. Specifically, all three gingipains (HRgpA, RgpB, and Kgp) act in a C5 convertase-like manner and generate biologically active C5a through limited degradation of C5, whereas the C5b remnant is functionally inert (125) (161). When C5 is oxidized by hydroxyl radicals (as may occur in the oxidative environment of the inflammatory response) the gingipains generate increased C5a biological activity (31). Furthermore, *P. gingivalis* may indirectly generate functional C5a by exploiting the physiological crosstalk between the



coagulation and the complement systems, which activates the so-called extrinsic pathway (66) (Fig. 1). Indeed, HRgpA and RgpB activate pro-thrombin to form thrombin (68) which, in turn, generates biologically active C5a by acting as a C5 convertase (66) (Fig. 1). Although C5a can potentially play a key role in host defense against infection (46), it seems highly unlikely that *P. gingivalis* uses its enzymes to generate C5a to contribute to its elimination.

An intriguing question, therefore, is whether there is any selective pressure or advantage for *P. gingivalis* to specifically generate C5a, given that this chronically persisting pathogen overall inhibits the complement cascade. A possible scenario is that local generation of excessive levels of C5a could incapacitate the antimicrobial function of gingival crevicular neutrophils rendering them less threatening to *P. gingivalis*. This is because neutrophils become immunologically paralyzed in the presence of high concentrations (10-100 nM) of C5a and thereby fail to carry out functions such as chemotaxis, phagocytosis, and production of antimicrobial and inflammatory mediators (67) (160). Such immunological dysfunction has been seen both *in vitro* and *in vivo* and involves both human and rodent neutrophils (67) (132) (160). In fact, C5a-mediated inhibition of neutrophil killing of *P. gingivalis* does occur, both *in vitro* and *in vivo* (J. Krauss and G. Hajishengallis, unpublished data). However, the underlying mechanisms, whether involving immune paralysis or alteration of specific signaling pathways, are currently under investigation. In addition to its potential exploitation by *P. gingivalis*, C5a may amplify periodontal tissue damage through its ability to recruit and activate inflammatory cells. For example, enhanced production of reactive oxygen species by C5a-stimulated neutrophils (46) may contribute to oxidative periodontal tissue



destruction (12). On the other hand, this host response would not affect *P. gingivalis*, since it is resistant to killing by reactive oxygen species (55) (111).

Even if the gingipain activity of *P. gingivalis* is capable of increasing the microenvironmental C5a concentrations to paralyzing levels for the neutrophils, this would not impinge on the function of macrophages, which can also be recruited to the gingival crevice or additionally interact with the pathogen in the periodontal connective tissue (28) (150). Indeed, macrophages are quite resistant to the deleterious effects of high C5a concentrations, because they express relatively modest levels of the C5a receptor relative to neutrophils (160). For instance, whereas the ability of neutrophils to induce tumor necrosis factor- $\alpha$  (and other innate responses) is inhibited in the presence of C5a at  $\geq 10$  nM, the macrophages display potentiated tumor necrosis factor- $\alpha$  responses under the same C5a concentrations (67) (132).

Therefore, even at high levels, C5a does not exert a general immunosuppressive influence on macrophages. Strikingly, however, C5a can specifically downregulate cytokines of the interleukin-12 family. Indeed, C5a-induced signaling in macrophages interferes with TLR-induced expression of mRNA for the interleukin-12 p35, interleukin-12/interleukin-23 p40, and interleukin-23 p19 subunits (63) (85). These regulatory effects are possibly mediated through C5a-induced phosphatidylinositol-3 kinase and extracellular signal-regulated kinase 1/2 signaling, which in concert suppress critical transcription factors, the interferon regulatory factor-1 and -8 (63) (Fig. 4). At the protein level, the production of interleukin-12 is inhibited both *in vitro* and *in vivo*, leading to suppression of T-helper type 1 cell-mediated immunity (63) (165). Moreover, the ability of C5a to inhibit mRNA expression of both interleukin-23 subunits strongly suggests that

C5a can interfere with the capacity of this cytokine to support the development of the T-helper type 17 cell subset (9). The physiological significance of these C5a regulatory effects is likely to attenuate potential tissue damage mediated by T-helper type 1 and T-helper type 17 cells, as seen in various pathological inflammatory conditions (37) (95) (138). However, undesirable outcomes may arise when C5a is not produced physiologically but through the uncontrolled action of microbial enzymes, such as the *P. gingivalis* gingipains. Since interleukin-12 is important for immune control of *P. gingivalis* (53), it is possible that this pathogen may exploit the C5a-induced crosstalk with TLR2 for inhibiting IL-12-dependent immune clearance. Such evasion mechanism may be complementary, rather than redundant, since the interaction of *P. gingivalis* with complement receptor-3 causes partial inhibition of interleukin-12 production (about 60%) (53). The notion that *P. gingivalis* hijacks C5a for its own benefit is additionally supported by observations that the intracellular survival of this pathogen in macrophages is promoted in the presence of C5a (157).

Interestingly, unlike C5a, C3a is extensively degraded by *P. gingivalis* gingipains and does not retain biological activity (161). Whether this is beneficial for the pathogen is uncertain, but it should be noted that C3a exerts direct antimicrobial effects and readily kills both gram-negative and gram-positive bacteria such as *E. coli*, *Pseudomonas aeruginosa*, and *Enterococcus faecalis* (114). If C3a can kill *P. gingivalis* as well, then its gingipain-mediated inactivation would serve to protect *P. gingivalis*.

In summary, it appears that *P. gingivalis* does not have a purely defensive agenda in dealing with the complement system. In other words, the pathogen may not restrict its action to simply inhibiting the complement cascade, but rather may proactively employ

specific complement components (such as the complement receptor-3 and the C5a) for bidirectional crosstalk interactions with TLR2 that favor the pathogen (Fig. 4). Therefore, we hypothesized that *P. gingivalis* can modulate TLR2 signaling by co-opting C5a receptor to instigate a subversive crosstalk that promotes its adaptive fitness. We speculated this may involve blunting the killing efforts of recruited leukocytes, without causing a wholesale immunosuppression. Moreover, this pathogen-induced crosstalk may serve to amplify select aspects of the inflammatory response, thereby liberating essential peptide nutrients essential for growth without promoting the antimicrobial defenses of recruited leukocytes that would likely facilitate its destruction.

## CHAPTER TWO: MATERIALS AND METHODS

### Materials and reagents

Carboxy methyl cellulose (CMC) was purchase from MP Biomedicals, Solon, OH. SQ22536, H89, SB216367, 8-Br-cAMP, AMD3100, forskolin, L-NAME (*N*(G)-nitro-L-arginine methyl ester), D-NAME (*N*(G)-nitro-D-arginine methyl ester), and EGTA were purchased from Sigma-Aldrich. Chelelythrin, PKI 6-22, KT5823, and thapsigargin were obtained from Calbiochem. PD98059 was from Cell Signaling Technology. Mouse-specific monoclonal antibodies to TLR2 [clone 6C2] was from e-Bioscience, TLR5 [85B152.5] from Abcam, and C5aR (20/70) from Cedarlane Laboratories or Hycult. Mouse rIFN- $\gamma$  was from the R&D Systems. Mouse rC5a was purchased from R&D Systems and rC3a from Cell Sciences. The cyclic hexapeptide AcF(OP(D)ChaWR) (acetylated phenylalanine-(ornithine-proline-(D) cyclohexylalanine-tryptophan-arginine), also known as PMX-53, is a specific and potent C5a receptor (CD88) antagonist, was synthesized in the laboratory of John D. Lambris, as previously described (35) (98). A8 <sup>$\Delta$ 71-73</sup>, also a generous gift of John D. Lambris, a dual antagonist of both C5aR and C5a-like receptor-2, was generated essentially as previously described (49). Specifically, the A8 <sup>$\Delta$ 71-73</sup> sequence (49) was created by three cycles of mutagenesis of the original human C5a construct (47), using the QuickChange XL Site-Directed Mutagenesis Kit from Stratagene. The three pairs of complementary primers used for mutagenesis are as follows (forward sequences given):

1) 5'-GTTACGATGGAGCCGCCGTTAATAATGATG-3',

2) 5'- CCGTGCTAATATCTCTTTTAAACGCATGCAATTGGGAAGG-3',

3) 5'-CTCTTTTAAACGCTCGTGAAAGCTTAATTAGC-3',

corresponding to mutations 1) C27A, 2) H67F and D69R, and 3) M70S and  $\Delta(71-74)$ , respectively. The protein was then expressed and purified as previously described (47). All reagents were used at optimal concentrations determined in preliminary or published studies by our laboratories (36) (47) (59) (113) (165). C5a and C3a were used at concentrations up to 50 nM and 100 nM, respectively, which are widely used for *in vitro* experiments (63) (165). Moreover, these concentrations are consistent with observations that under inflammatory conditions, C5a and C3a may reach serum levels as high as 100 nM and 400 nM, respectively, although even higher levels may be generated at local sites of inflammation (149) (160). All reagents were used at optimal concentrations determined in preliminary or published studies by our laboratories (59) (94) (98). When appropriate, dimethyl sulfoxide (DMSO) was included in medium controls and its final concentration was  $\leq 0.2$  %.

### **Animals**

Both female BALB/c and C57BL/6 C5aR-deficient mice (with their respective wild-type controls) were used in these studies. The TLR2-deficient mice were originally C57BL/6 (The Jackson Laboratory) and we backcrossed them for nine generations onto a BALB/c genetic background prior to use in these studies. The C5aR-deficient mice were

originally obtained from Dr. Craig Gerard (Harvard Medical School) and are now housed at The Jackson Laboratory. All animal procedures were approved by the Institutional Animal Care and Use Committee and performed in compliance with established federal and state policies.

### **Culturing of bacterial strains**

*P. gingivalis* ATCC 33277 and its isogenic KDP128 mutant, which is deficient in all three gingipain genes (*rgpA*, *rgpB*, and *kgp*) (45) (kindly provided by Dr. K. Nakayama, Nagasaki University, Japan), were grown anaerobically from frozen stocks on modified Gifu anaerobic medium-based blood agar plates for 5-6 days at 37°C, followed by anaerobic subculturing for 18-24 hours at 37°C in modified Gifu anaerobic medium broth (Nissui Pharmaceutical).

### **Oral gavage model**

Oral infection of mice proceeded as previously described by Baker et al (8). In brief, Balb/c mice were provided antibiotic-supplemented drinking water (800 mg sulfamethoxazole and 400 mg trimethoprim per liter of water) for 10 days prior to experiment. Following a 3 day regimen of antibiotic-free water, mice were orally infected with a  $10^9$  suspension in 2% CMC in PBS of *P. gingivalis*, repeated every other day for a total of 5 inoculations. Six weeks following the final inoculation, total anaerobic counts of bacteria were enumerated from paper-point samples grown under anaerobic conditions on blood agar plates for 7 days. Moreover, periodontal bone loss was determined by subtracting the measured distance from the cemento-enamel junction

(CEJ) to the alveolar bone crest (ABC) of *P. gingivalis*-infected from the group of sham-infected mice. Results are expressed as mm change in bone loss.

### **Subcutaneous chamber model**

Isoflurane anesthetized Balb/c mice were dually implanted with a surgical-grade titanium coil chamber. Following a 7 day healing period, *P. gingivalis* ( $10^9$  CFU in 100  $\mu$ l of PBS) was injected into the chambers of each mouse. Chamber exudates were harvested from mice at indicated time points and centrifuged at 1000 rpm for 5 minutes. Subsequently, recruited cells were phenotypically characterized by flow cytometry and supernatants were used to determine viable counts of *P. gingivalis*.

### **Intracellular survival assay in murine macrophages**

Thioglycollate-elicited macrophages were isolated from the peritoneal cavity of wild-type or mice deficient in TLR2, TLR4, C3aR, or C5aR (The Jackson Laboratory) (54) (165), in compliance with established federal guidelines and institutional policies. The macrophages were cultured at 37°C and 5% CO<sub>2</sub> in RPMI 1640 (Invitrogen) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 units/ml penicillin G, 100  $\mu$ g/ml streptomycin, and 0.05 mM 2-ME. None of the experimental treatments, including treatments with C5a up to 100 nM, affected cell viability (monitored by the CellTiter-Blue™ assay; Promega) compared to medium-only treatments. The viability of phagocytosed *P. gingivalis* was monitored by an antibiotic protection-based intracellular survival assay, as previously described (158). Briefly, mouse peritoneal macrophages were allowed to phagocytose *P. gingivalis* (MOI = 10:1;  $5 \times 10^6$  bacteria and  $5 \times 10^5$  cells) for 30 min at 37°C. This was followed by washing to remove extracellular non-

adherent bacteria and 1-hour treatment with antibiotics (300 µg/ml gentamicin and 200 µg/ml metronidazole) to eliminate residual or extracellular adherent bacteria. The macrophages were subsequently cultured overnight (for a total of 24 hours) or for 48 hours. Immediately after, the macrophages were washed and lysed in sterile distilled water and viable counts of internalized *P. gingivalis* were determined by plating serial dilutions of macrophage lysates on blood agar plates subjected to anaerobic culture (158).

### **Cell signaling and activation assays**

Induction of nitric oxide production was assessed by measuring the amount of  $\text{NO}_2^-$  (stable metabolite of nitric oxide) in stimulated culture supernatants using a Griess reaction-based assay kit (R&D Systems), as previously performed (59). Levels of cAMP in activated cell extracts were measured using a cAMP enzyme immunoassay kit (Cayman Chemical) (94). PKA activity in lysates of activated cells was determined using the ProFluor™ PKA assay, according to the instructions of the manufacturer (Promega) (59). Phosphorylation of GSK3β on Ser9 and total GSK3β were monitored using FACE™ GSK3β ELISA kits (Active Motif).

### **Intraperitoneal infection (i.p.)**

Upon i.p. infection of mice with *P. gingivalis* ( $5 \times 10^7$  CFU), peritoneal lavage was performed 24 hours post-infection and the peritoneal fluid was used to enumerate recovered CFU (following anaerobic growth on blood agar plates) and measure production of  $\text{NO}_2^-$  (59).



### **Quantitative real-time PCR**

Gene expression in resting or activated mouse macrophages was quantified using quantitative real-time PCR. Briefly, RNA was extracted from cell lysates using the PerfectPure RNA cell kit (5 Prime, Fisher) and quantified by spectrometry at 260 and 280 nm. The RNA was reverse-transcribed using the High-Capacity cDNA Archive kit (Applied Biosystems) and quantitative real-time PCR with cDNA was performed using the ABI 7500 Fast System, according to the manufacturer's protocol (Applied Biosystems). TaqMan probes, sense primers, and antisense primers for expression of a house-keeping gene (GAPDH) or iNOS) were purchased from Applied Biosystems.

### **Confocal microscopy**

To examine co-localization of *P. gingivalis* with C5aR and TLR2, mouse macrophages were grown on chamber slides and exposed to FITC-labeled *P. gingivalis* for 10 min. The cells were then fixed, permeabilized, stained with Texas Red-labeled anti-C5aR plus allophycocyanin-labeled anti-TLR2, and mounted with coverslips for imaging on an Olympus FV500 confocal microscope (158).

### **Fluorescence resonance energy transfer (FRET)**

Upon stimulation for 10 min at 37°C with *P. gingivalis*, mouse macrophages were labeled with a mixture of Cy3-conjugated (donor) and Cy5-conjugated (acceptor) antibodies. In other experiments shown in Fig. 13A, FITC-labeled *P. gingivalis* was used as donor and TRITC-labeled receptors served as acceptors. The cells were washed and fixed, and energy transfer between various donor-acceptor pairs was calculated from the increase in donor fluorescence after acceptor photobleaching (54) (152). The maximum

(max) and minimum (min) energy transfer efficiencies in the experimental system were determined in control experiments as the energy transfer between two different epitopes on the same molecule or between molecules that do not engage in heterotypic associations, and their values are denoted by dashed lines in Fig. 13A. The conjugation of antibodies to Cy3 or Cy5 was performed using kits from Amersham Biosciences

### **Statistical analysis**

Data were evaluated by analysis of variance and the Dunnett multiple-comparison test using the InStat program (GraphPad Software, San Diego, CA). Where appropriate (comparison of two groups only), two-tailed *t* tests were performed.  $P < 0.05$  was taken as the level of significance. All experiments were performed at least twice for verification.

## CHAPTER THREE: MOUSE MODELS OF PERIODONTAL HOST-PATHOGEN INTERACTIONS AND INFLAMMATION

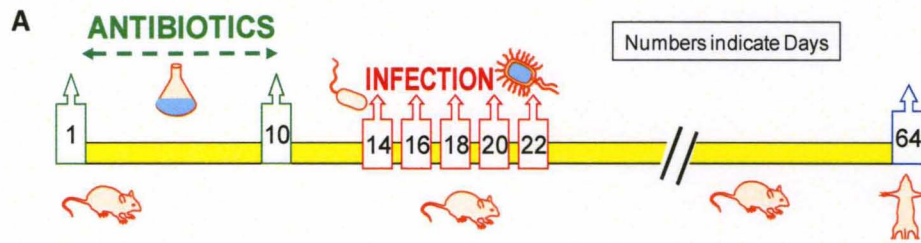
### Introduction

Periodontitis is a prevalent chronic inflammatory disease that affects the tooth-supporting tissues and can exert a systemic impact on health (73) (123) (151). A group of tooth-associated subgingival anaerobic bacteria is strongly associated with periodontitis (146), however, it is the host inflammatory response to uncontrolled bacterial challenge that primarily mediates periodontal tissue destruction (37) (43) (72). Although no single animal model can faithfully reproduce all aspects of periodontitis (or any other disease), the power and significance of animal models involves their capacity to test specific hypotheses involving distinct aspects of periodontal pathogenesis (44). Knowledge gathered from different but complementary models can be synthesized appropriately to obtain unique insights into the mechanisms of periodontitis. Despite their limitations, animal models are absolutely necessary for determining cause-and-effect relationships and for assessing the potential of novel therapeutic compounds. Such studies cannot be adequately served by *in vitro* experiments. Moreover, causal mechanistic relationships cannot normally be addressed in human studies which are often correlative in nature (44). It should also be noted that clinical trials can be initiated only

after safety and efficacy has been demonstrated in animal models. As models of human disease, mice provide unique advantages beyond their relatively low cost and ease of handling. These include extensive background information on their immune system and a wide range of immunologic and cellular/molecular reagents. Moreover, the availability of a host of transgenic mouse lines can be used to study the impact of specific immunoregulatory genes.

### **Inflammatory periodontal bone loss: the oral gavage model**

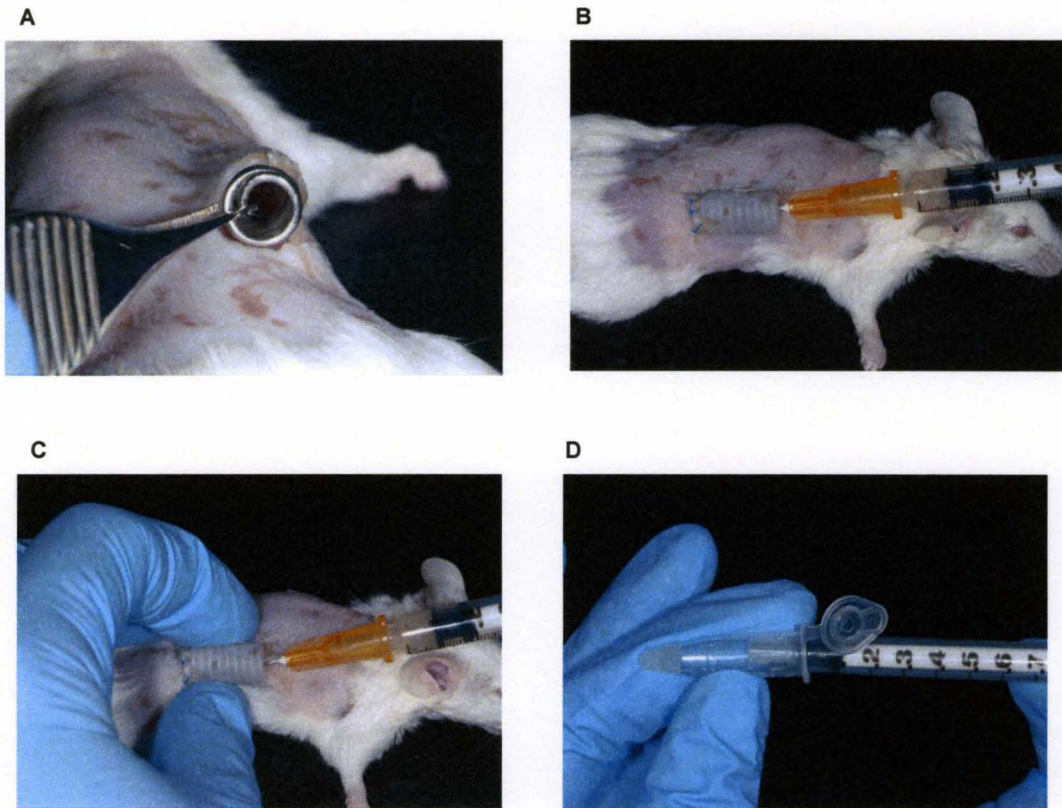
A mouse periodontitis model was developed by Baker and colleagues (6) (7) (8) ('Baker' or 'Oral gavage' model) and is now widely used with various modifications. Reproducible gingival inflammation and alveolar bone loss can be induced in this model following oral gavage with *Porphyromonas gingivalis* (7) (158) or other periodontal pathogens, such as *Aggregatibacter actinomycetemcomitans* (38) (115), *Tannerella forsythia* (140) or *Porphyromonas gulae*, an animal periopathogen equivalent to human *P. gingivalis* (60). The model can be further modified to involve a co-infection, e.g., *P. gingivalis* and *Fusobacterium nucleatum* (78) (124). Additionally, the oral gavage model of periodontitis has been used for rapid and cost-effective identification of pathogenic mechanisms and potential therapeutics (7) (38) (42) (53) (86) (115). Specifically, these mouse studies have helped determine the role of defined innate receptors or cytokines in periodontal tissue destruction, substantiate a genetic basis for host susceptibility or resistance to periodontal disease, identify virulence factors and evasion strategies of periodontal pathogens, and offer potential mechanistic links between periodontal and systemic diseases (44).



**Figure 5. Oral gavage model of *P. gingivalis*-induced mouse periodontitis.** (A) Timeline of the experimental protocol. (B) Oral inoculation of mice with *P. gingivalis* using a ball-ended feeding needle. The mouse is restrained by grasping the skin over the shoulders and holding the tail.

### **Host cell-periodontal bacteria interactions: the subcutaneous chamber model**

A widely used mouse model to study host-pathogen interactions is the so-called chamber model, which was introduced to periodontal research by Genco and colleagues (39). The chamber comprises a coil-shaped titanium wire that is surgically implanted subcutaneously into the mid-dorsal region of the mouse. As the exterior encapsulates with connective tissue during the healing phase of implantation, the interior lumen becomes increasingly hypoxic (44), creating an environment reminiscent of the periodontal pocket. Oral bacteria can be injected into the chamber lumen and their interactions with recruited inflammatory cells can be assessed accurately and quantitatively (11) (39) (111). A major advantage of the chamber model is that some of these parameters, especially monitoring viable species-specific bacterial counts, are difficult to assess quantitatively in mucosal infection models. Additionally, inflammatory responses of recruited leukocytes can also be evaluated. Moreover, long-term versions of the chamber model can be used to monitor respective leukocyte populations recruited into the chamber or to study bacterial interactions over time. This model is thus appropriate for investigating interactions of periodontal bacteria with recruited inflammatory cells under conditions that faithfully mimic the subgingival environment.



**Figure 6. Subcutaneous chamber model.** (A) Subcutaneous implantation of surgical grade titanium-coil chamber. (B) Intrachamber injection of *P. gingivalis* ( $10^9$ ) following 7 day healing period. Aspiration (C) of chamber fluid, immediately following desired inoculation period, and collection (D) for further analysis.

## **Results**

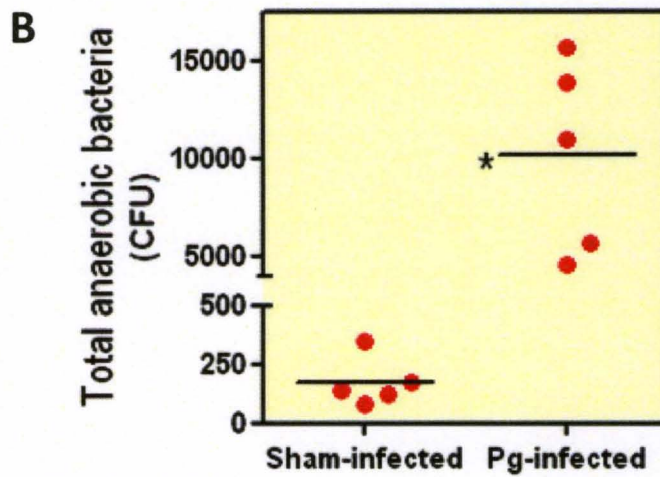
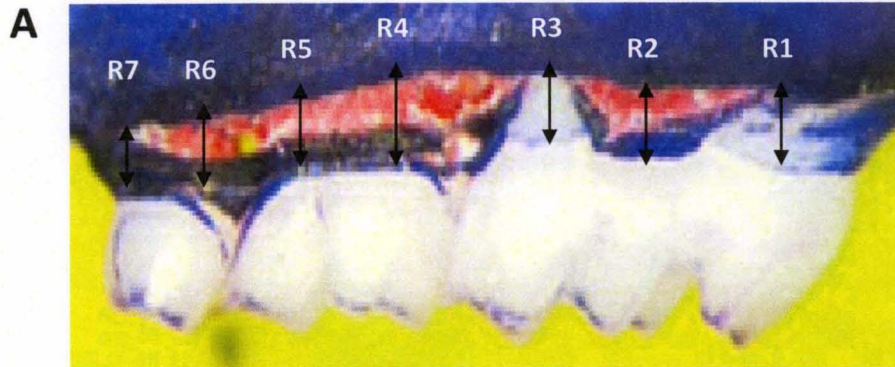
### ***P. gingivalis* augments the population of indigenous microflora in the oral gavage model**

To ascertain the effects of oral challenge with *P. gingivalis* (Pg) on the recovery of indigenous anaerobic bacteria residing within the oral cavity, total colony forming-units (CFUs) were enumerated and contrasted to CFUs recovered from sham-infected mice (Fig. 7B). Our results clearly demonstrate that the presence of *P. gingivalis* (Pg) significantly augments the population size of resident oral anaerobic bacteria recovered by paper-point sampling from the oral cavity compared to sham-infected control mice. Indeed, the co-presence of *P. gingivalis* (Pg) within the periodontium potentiated the overall number of indigenous oral microflora by two orders of magnitude.

### **Neutrophils are impaired in their ability to promote the clearance of *P. gingivalis* in the subcutaneous chamber model**

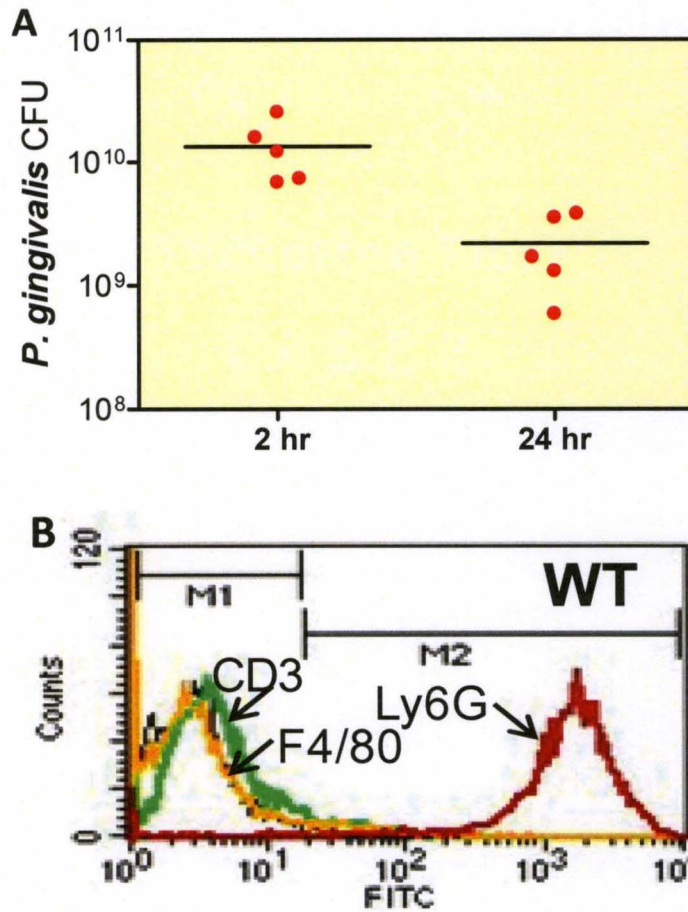
Using the subcutaneous chamber model, we monitored inflammatory cell recruitment and investigated the fate of *P. gingivalis* within an *in vivo* setting that closely mimics the environment of the periodontal crevice. We observed that the overwhelming majority of cells recruited in response to intrachamber challenge with *P. gingivalis* were neutrophils (>97% at 24h post-infection) (Fig. 8B). However, we found that despite a massive influx into the chamber, neutrophils were impaired in their ability to adequately clear the infection, observed at either early or late time points (Fig. 8A).





**Figure 7. Recovery of total anaerobic bacteria from oral gavage model.**

(A) Pre-determined buccal sites in the right maxilla (R1-R7) for measuring CEJ-ABC distances. (B) Oral infection with *P. gingivalis* (Pg) causes major increases in the numbers of the indigenous oral anaerobic bacteria ( $p < 0.01$  vs. sham-infected mice) recovered by paper-point sampling.



**Figure 8. Analysis of chamber exudates from *P. gingivalis*-challenged subcutaneous chamber model.** (A) Recovery of viable *P. gingivalis* colony-forming units (CFUs) at 2 and 24 hours post-intrachamber challenge. (B) Phenotypic characterization [CD3 (green), F4/80 (yellow) and Ly6G (red)] of recruited leukocytes 24 hours post-intrachamber infection with *P. gingivalis*.

## DISCUSSION

The bimodal nature of periodontal disease requires an experimental system that can equally address both host and bacterial factors (mutually inclusive contributors) that promote periodontal tissue destruction. Due to its experimental versatility, the mouse oral gavage model, an *in vivo* experimental system (7), is a time-honored and faithful rendition of periodontal disease. For example, through the use of genetically altered mice, the oral gavage model can be employed to monitor changes in gene expression within the gingival tissues as well as quantify appreciable bone loss in the presence of keynote periodontal pathogens, such as *Porphyromonas gingivalis* (7) (8) (44) (58). In addition to examining host determinants that contribute to periodontal disease, microbial counts can also be enumerated. Intriguingly, through the use of this *in vivo* model, we demonstrate that the presence of *P. gingivalis* can stimulate a substantial rise in the overall numbers of indigenous oral anaerobic bacteria residing within the oral cavity (Fig. 7B). These findings suggest that alterations in the oral microbial profile correspond with the prevalence of *P. gingivalis*, underscoring its importance for potentially promoting survival and virulence of the entire microbial community (21) (22) (49) (82).

The dichotomy of both protective and destructive immunity in the periodontium highlights the critical importance of discerning the precise roles leukocytes play with regards to periodontal disease. Although well-accepted and heavily utilized, the oral gavage model offers a rather panoramic readout of mucosal responses to infection. However, the subcutaneous chamber model provides an alternative *in vivo* model that can be utilized to determine specific host and bacterial factors driving both protective and destructive aspects of periodontal disease. Our studies reveal that in response to

challenge with *P. gingivalis*, the lumen of the subcutaneous chamber becomes predominated almost exclusively by neutrophils (Fig. 8B), similar to an environment encountered within the gingival crevice, where  $\geq 95\%$  of total leukocytes are indeed neutrophils (28). Strikingly, *P. gingivalis* can survive and persist within the chamber although high numbers of neutrophils are chemoattracted there (Fig. 8A). These findings suggest that neutrophils may not be particularly adept at clearing *P. gingivalis* from the gingival crevice and may, in fact, play a destructive rather than protective role in periodontitis.

In general, the strength of the chamber model involves the whole-animal aspect for accurately quantifiable host responses or microbiological parameters (inflammatory cell recruitment, cytokine responses, bacterial clearance or persistence, and so on) (11) (39) (44). Since the significance of animal models involves their capacity to test defined concepts or hypotheses, rather than their fidelity to all aspects of a given disease (44), the chamber model is suitable to determine how specific host response genes or putative virulence factors shape the outcome of host interactions with periodontal bacteria (28) (111). Therefore, the chamber model can complement the oral gavage model for a more complete and nuanced understanding of the periodontal host-pathogen interplay.

## CHAPTER FOUR: MICROBIAL HIJACKING OF COMPLEMENT-TOLL-LIKE RECEPTOR CROSSTALK

### Introduction

Although traditionally perceived as an antimicrobial enzyme system in serum, complement is now recognized as a central component of host defense impacting both innate and adaptive immunity (99). More recently, complement was suggested to crosstalk with another major innate defense system, the Toll-like receptors (TLRs), to apparently coordinate the host response to infection (52) (63). Not surprisingly, given its importance in fighting pathogens, complement constitutes a key target of immune evasion by microbes which cause persisting infections (52). Here we describe a novel strategy of immune subversion, involving microbial exploitation of the fifth complement component (C5) for corrupting TLR immunity via a hitherto unknown mechanism of complement-TLR crosstalk.

The pathogen involved in these subversive interactions, *Porphyromonas gingivalis*, is a gram-negative anaerobic bacterium. This organism is strongly associated with periodontitis, a highly prevalent oral chronic inflammatory disease, and is moreover implicated in systemic conditions such as atherosclerosis and aspiration pneumonia (10). Although *P. gingivalis* inhibits the overall complement cascade regardless of the initiation pathway involved, curiously enough, this pathogen selectively generates biologically active C5a (69) (116). C5a generation by *P. gingivalis* is mediated by its

Arg-specific cysteine proteinases (HRgpA and RgpB gingipains) which act in a C5 convertase-like manner (69) (116). Interestingly, upon release of C5a from C5, the C5b remnant is proteolytically destroyed by *P. gingivalis* (6) to apparently prevent activation of the terminal complement pathway, which leads to the formation of the membrane attack complex (99). Since C5a is a powerful chemoattractant and activator of phagocytes (88), it seems counterproductive for a pathogen to actively contribute to C5a generation. An intriguing question, therefore, is whether there is any survival advantage for *P. gingivalis* to specifically generate C5a in its periodontal niche, where complement proteins are abundantly present at up to 70% of their concentration in serum (116).

Below we present evidence that *P. gingivalis* paradoxically employs the pro-inflammatory C5a for targeted immune suppression of macrophages through a novel crosstalk mechanism between the C5a receptor (C5aR) and TLR2, the predominant TLR utilized by this organism *in vitro* and *in vivo* (123) (137). This is the first report for a pathogen capable of proactively instigating and exploiting crosstalk signaling between complement and TLRs, rather than undermining either system independently as previously shown for a number of other microbes (52) (161).

## **Results**

### **C5a and subversion of macrophage function**

We were prompted to investigate whether C5a signaling is advantageous to *P. gingivalis* by earlier observations that its enzymatic activity selectively generates functional C5a, despite overall inhibiting the complement cascade (69) (116). We first examined whether C5a influences the macrophage intracellular killing of *P. gingivalis*.

Strikingly, the ability of this pathogen to survive intracellularly in mouse macrophages was significantly promoted by C5a, but not by the related anaphylatoxin C3a (Fig.9, A and B). This unexpected pro-microbial effect of C5a was also observed in interferon (IFN)- $\gamma$ -primed macrophages (Fig. 9, C and D). The elevated viable cell counts of *P. gingivalis* in C5a-treated macrophages could not be attributed to possible differences in the initial bacterial loads, since *P. gingivalis* phagocytosis was not significantly affected by the absence or presence of C5a or C3a (data not shown).

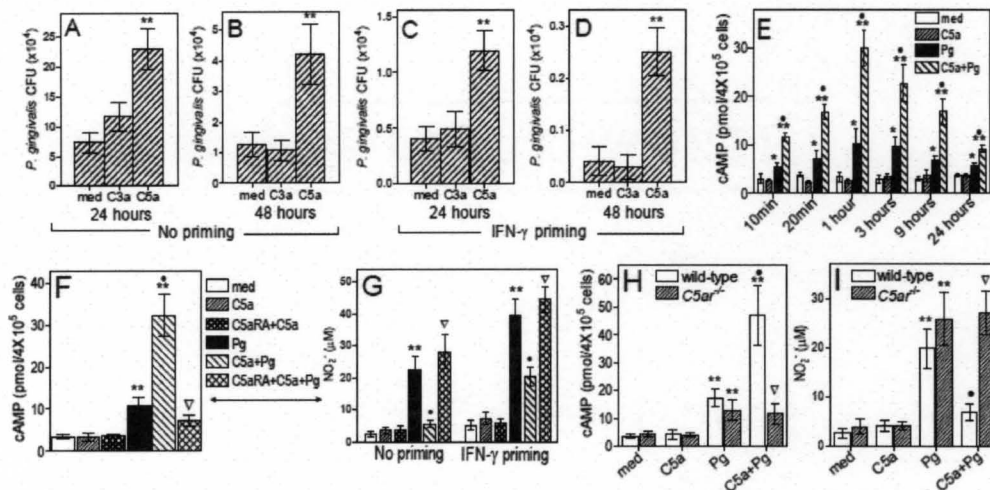
We next investigated the mechanism(s) underlying C5a-mediated inhibition of the macrophage intracellular killing capacity. In this regard, we hypothesized that the combined action of C5a and *P. gingivalis* on macrophages may induce immunosuppressive signaling. We first used real-time quantitative PCR to determine whether C5a upregulates the expression of negative regulators of TLR signaling in *P. gingivalis*-stimulated macrophages. Although the bacterium alone upregulated the expression of some of the investigated regulators, including the suppressor of cytokine signaling-1, the interleukin-1 receptor-associated kinase M, and the ubiquitin-editing enzyme A20, no synergistic or additive effects were seen in the concomitant presence of *P. gingivalis* and C5a (data not shown). Therefore, these regulatory molecules are not likely involved in C5a-mediated suppression of macrophage killing of *P. gingivalis*. Moreover, although induction of cAMP can induce immunosuppressive signaling (12), C5a by itself failed to induce a cAMP response in macrophages (Fig. 9E). Strikingly, however, C5a synergized with *P. gingivalis* resulting in >3-fold elevation of the intracellular cAMP levels relative to *P. gingivalis* stimulation alone (Fig. 9E). The

synergy was observed as early as 10 min after cell stimulation, peaked at 1 hour, but significantly elevated cAMP levels were sustained for at least 24 hours (Fig. 9E).

This upregulatory effect of C5a was dose-dependent (data not shown) and was totally abrogated by a C5aR antagonist (C5aRA), the cyclic hexapeptide AcF(OP(D)ChaWR) (Fig. 9F), indicating that C5a acted through the classic C5aR (CD88), rather than the alternative C5a-like receptor 2 (C5L2).

Given that *P. gingivalis* is exquisitely resistant to killing by the oxidative burst (160), we investigated whether C5a interferes with induction of nitric oxide as a possible mechanism for its pro-microbial effect. The underlying rationale was that *P. gingivalis* is sensitive to nitric oxide-mediated killing (11) (54) . Indeed, C5a significantly inhibited, via a C5aR-dependent mechanism, the production of nitric oxide in *P. gingivalis*-stimulated macrophages, even in cells primed with IFN- $\gamma$  (Fig. 9G). The C5aR specificity of the C5a-driven augmentation of cAMP and suppression of nitric oxide in *P. gingivalis*-challenged macrophages was confirmed by lack of these effects in C5aR-deficient (*C5ar*<sup>-/-</sup>) macrophages (Fig. 9, H and I, respectively). *In toto*, our findings suggest that C5aR activation by C5a results in suppression of *P. gingivalis* intracellular killing associated with elevation of cAMP and reduction of nitric oxide.

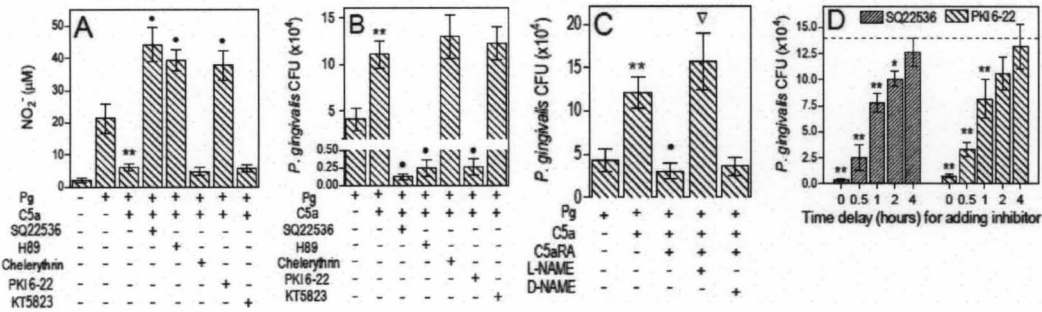




**Figure 9. Immunosubversive effects of C5a on *P. gingivalis*-challenged macrophages.** Untreated (A, B) or IFN- $\gamma$  primed (C, D) macrophages were incubated with *P. gingivalis* in the presence or absence of C3a (200 nM) or C5a (50 nM). Colony counts of internalized bacteria were enumerated at 24 (A, C) or 48 hours (B, D) post-infection (E) *P. gingivalis*-challenged macrophages, in the absence or presence of C5a, were assayed for intracellular cAMP production for the times indicated. (F) Similar experiment as in (E), involving 1 hour of incubation and the use of a specific C5a receptor antagonist (C5aRA; 1  $\mu$ M). (G) Unprimed or IFN- $\gamma$ -primed macrophages were assayed for NO<sub>2</sub><sup>-</sup> production after incubation with or without *P. gingivalis*, C5a, or C5aRA. Induction of cAMP (H) and NO<sub>2</sub><sup>-</sup> (I) production with macrophages from wild-type or C5aR-deficient (*C5ar*<sup>-/-</sup>) mice. Data are means  $\pm$  SD (n = 3) from typical experiments performed three (A-D, F and G) or two (E, H, and I) times yielding consistent results. \*P < 0.05 and \*\*P < 0.01 compared to medium only treatments. •P < 0.01 in C5a + Pg compared to Pg alone. Inverted triangles indicate significant (P < 0.01) reversal of C5a effects by C5aRA or C5aR deficiency.

### **C5a immunosubversive effects are strictly dependent on cAMP-PKA signaling**

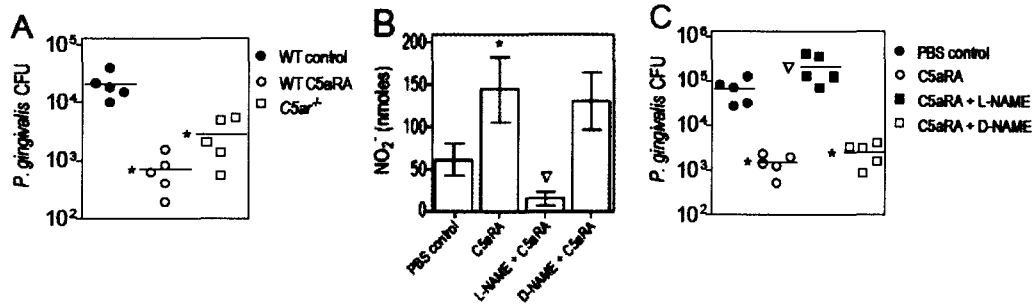
We investigated whether the C5a-mediated inhibition of nitric oxide production depends upon the ability of C5a to stimulate synergistic elevation of cAMP. Indeed, the inhibitory C5a effect on nitric oxide was reversed in macrophages pretreated with inhibitors of cAMP synthesis (SQ22536) or of PKA (H89 and PKI 6-22) but not of irrelevant kinases (chelerythrin or KT5823) (Fig. 10A), indicating that the C5a effect is mediated by cAMP-dependent PKA signaling. Importantly, the upregulation of nitric oxide levels by inhibitors of cAMP or of PKA was linked to significantly reduced intracellular survival of *P. gingivalis* in those same cells (Fig. 10B). Moreover, macrophage pretreatment with C5aRA counteracted the protective effect of C5a on *P. gingivalis* intracellular viability, whereas L-NAME (nitric oxide synthesis inhibitor) mimicked C5a and overrode the C5aRA effect (Fig. 10C). In contrast, D-NAME, an inactive enantiomer control, had no effect in that regard (Fig. 10C). Interestingly, the ability of inhibitors of cAMP or of PKA to reverse the immunosuppressive C5a effect progressively declined with increasing delay of their addition to the culture system (Fig. 10D). Therefore, *P. gingivalis* needs to immediately activate cAMP-dependent PKA signaling to suppress the macrophage killing capacity, consistent with the requirement for early availability of C5a in order to disable *P. gingivalis*-challenged macrophages.



**Figure 10. C5a-mediated inhibition of nitric oxide production and promotion of *P. gingivalis* survival is cAMP and PKA dependent.** (A, B) Mouse macrophages were pretreated or not with SQ22536 (cAMP synthesis inhibitor), H89 (PKA inhibitor), chelerythrin (protein kinase C inhibitor), PKI 6-22 (peptide inhibitor of PKA), or KT5823 (peptide inhibitor of protein kinase G), and then infected with *P. gingivalis* with or without C5a. (C) Macrophages were pretreated with L-NAME (or D-NAME), C5aRA, or both and then infected with *P. gingivalis* with or without C5a. (D) Macrophages were incubated with *P. gingivalis* and C5a in the absence or presence of SQ22536 or PKI 6-22, added before *P. gingivalis* and C5a (“0 time delay”) or with increasing delay times, as indicated. NO<sub>2</sub><sup>-</sup> production (A) and viable counts of internalized bacteria (B-D) were determined at 24 hours after infection. In (D), the dashed line indicates *P. gingivalis* CFUs in the absence of inhibitors [ $13.7 \pm 2.7 (\times 10^4)$  CFUs]. Results are means  $\pm$ SD (n= 3) from typical experiments performed at least twice with consistent results. \*P < 0.05 and \*\*P < 0.01 compared to corresponding controls. •P < 0.01 in C5a + *Pg* with inhibitor or antagonist compared to C5a + *Pg* only. In (C), the inverted triangle shows significant (P < 0.01) reversal of the C5aRA effect.

### ***In vivo* exploitation of C5aR signaling for inhibition of nitric oxide and promotion of microbial survival**

To determine if C5aR signaling promotes *P. gingivalis* virulence also *in vivo*, we investigated the pathogen's ability to survive in mice after intraperitoneal infection, in the absence or presence of C5aRA. At 24 hours post-infection, the peritoneal lavage fluid from C5aRA-treated mice contained significantly lower *P. gingivalis* CFU compared to control mice (>95% reduction; Fig. 11A). Consistent with this, *C5ar<sup>-/-</sup>* mice were superior to wild-type controls in controlling the *P. gingivalis* infection (Fig. 11A). The wild-type control mice were additionally found to be bacteremic for *P. gingivalis* (4 out of 5 mice in this group had positive blood cultures 24 hours post-infection), whereas no bacteremia could be detected in *C5ar<sup>-/-</sup>* or C5aRA-treated wild-type mice, further indicating that C5aR signaling promotes *P. gingivalis* virulence. Additional support that the reduced peritoneal bacterial burden in the absence of C5aR signaling reflects increased *P. gingivalis* killing (rather than *P. gingivalis* escaping and taking up residence in internal organs) was obtained by lack of *P. gingivalis* CFU detection in homogenates of several organs examined (spleen, kidney, liver, and lungs) from either *C5ar<sup>-/-</sup>* or wild-type mice. The ability of C5aRA-treated mice for enhanced clearance of *P. gingivalis* correlated with elevated nitric oxide production (relative to control mice), whereas L-NAME counteracted both effects (Fig. 11, B and C). Therefore, as shown *in vitro*, the *in vivo* exploitation of C5aR signaling by *P. gingivalis* for enhanced survival involves a nitric oxide-dependent mechanism.



**Figure 11. *P. gingivalis* exploits C5aR signaling to inhibit nitric oxide production and promote its survival in vivo.** (A) Wild-type (WT) mice were intraperitoneally pre-treated with C5aRA (1 mg per kilogram body weight) or phosphate-buffered saline (PBS), followed by intraperitoneal infection of these mice, as well as mice deficient in C5aR (*C5ar*<sup>-/-</sup>), with *P. gingivalis* ( $5 \times 10^7$  CFU). Wild-type mice were intraperitoneally pre-treated or not with C5aRA, with or without L-NAME or D-NAME (0.1 ml of 12.5 mM solution, corresponding to 0.34 mg per mouse), followed by intraperitoneal infection with *P. gingivalis* (B and C). Peritoneal fluid was harvested 24 hours after infection and used to determine viable *P. gingivalis* CFU (A and C) and NO<sub>2</sub><sup>-</sup> production (B). Data are from typical experiments performed twice yielding consistent findings and represent means  $\pm$  SD (n = 5 mice) or are shown for each individual mouse with horizontal lines denoting mean values. \*P < 0.01 compared to controls. The inverted triangles show significant (P < 0.01) reversal of the C5aRA effects.

### **Synergistic activation of the cAMP-PKA pathway requires C5aR-TLR2 crosstalk**

A systematic analysis of crosstalk in intracellular signaling pathways has revealed that receptor-mediated elevation of intracellular  $\text{Ca}^{2+}$  may potentiate cAMP induction by appropriate stimuli (36). If the synergistic effect of C5a on cAMP induction (Fig. 9E) depends upon its  $\text{Ca}^{2+}$ -mobilizing activity, then this synergy should be inhibited by thapsigargin, an inhibitor of the endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase which blocks the C5a-induced intracellular  $\text{Ca}^{2+}$  response (121). Indeed, macrophage pretreatment with thapsigargin abrogated the synergistic C5a effect on *P. gingivalis*-induced cAMP, whereas EGTA, which chelates extracellular  $\text{Ca}^{2+}$ , had a relatively minimal and statistically insignificant effect (Fig. 12A). Significant reversal of the C5a effect on cAMP induction was also seen in cells pretreated with pertussis toxin (Fig. 12A), suggesting  $\text{G}\alpha_i$ -coupled C5aR signaling (49).

In the absence of C5a, the ability of *P. gingivalis* to induce cAMP depends on its interaction with the CXC-chemokine receptor 4 (CXCR4) (11). We thus initially speculated that the synergistic C5a effect on cAMP induction could involve a crosstalk between C5aR and CXCR4. Although CXCR4 blockade by AMD3100 (at 1  $\mu\text{g}/\text{ml}$  which completely inhibits the CXCR4-*P. gingivalis* interaction (11)) modestly attenuated the synergistic C5a effect on cAMP production, the synergism was still profoundly manifested (>6-fold difference between AMD+C5a+Pg vs. AMD+Pg; Fig. 12B). Moreover, *P. gingivalis* failed to elevate intracellular cAMP in CXCR4-transfected CHO-K1 cells, although it induced cAMP production in cells co-transfected with CXCR4 and TLR2 (data not shown). Therefore, CXCR4 is not directly involved in cAMP induction but cooperates in that regard with TLR2, which on its own induces a rather weak cAMP

response (data not shown). We next showed that the synergistic C5a effect on cAMP induction actually involves a crosstalk with TLR2.

Indeed, the ability of C5a to synergistically induce cAMP and activate PKA in *P. gingivalis*-stimulated wild-type macrophages was utterly absent in similarly stimulated *Tlr2*<sup>-/-</sup> macrophages, which displayed only background activity levels (Fig. 12, C and D). However, the inherent capacity of *Tlr2*<sup>-/-</sup> macrophages to elevate intracellular cAMP and activate PKA was confirmed by including a forskolin control (direct adenylate cyclase activator) (Fig. 12, C and D). This novel concept of C5aR-TLR2 crosstalk for synergistic cAMP-dependent PKA activation is consistent with additional findings from an *in vivo* experiment. Indeed, the PKA activity detected in freshly explanted peritoneal macrophages from *P. gingivalis*-infected mice was significantly reduced by TLR2 or C5aR deficiency, but not by TLR4 or C3aR deficiency, relative to cells from wild-type mice (Fig. 12E).

We also showed that another synergistic interaction downstream of this receptor crosstalk involved PKA-dependent phosphorylation of glycogen synthase kinase-3 $\beta$  (GSK3 $\beta$ ) on Ser9 (Fig. 12F), an event that inactivates this kinase which would otherwise positively regulate cell activation (47). Indeed, although C5a or *P. gingivalis* by themselves only slightly increased Ser9-phosphorylation of GSK3 $\beta$ , their combination displayed a synergistic effect which was inhibited by PKI 6-22 (but not by PD98059 control, an inhibitor of mitogen-activated protein kinase kinase) (Fig. 12F). Importantly, the GSK3 $\beta$  inhibitor SB216763 mimicked the inhibitory C5a effect on *P. gingivalis*-induced iNOS expression and nitric oxide production, as did 8-Br-cAMP (PKA agonist;

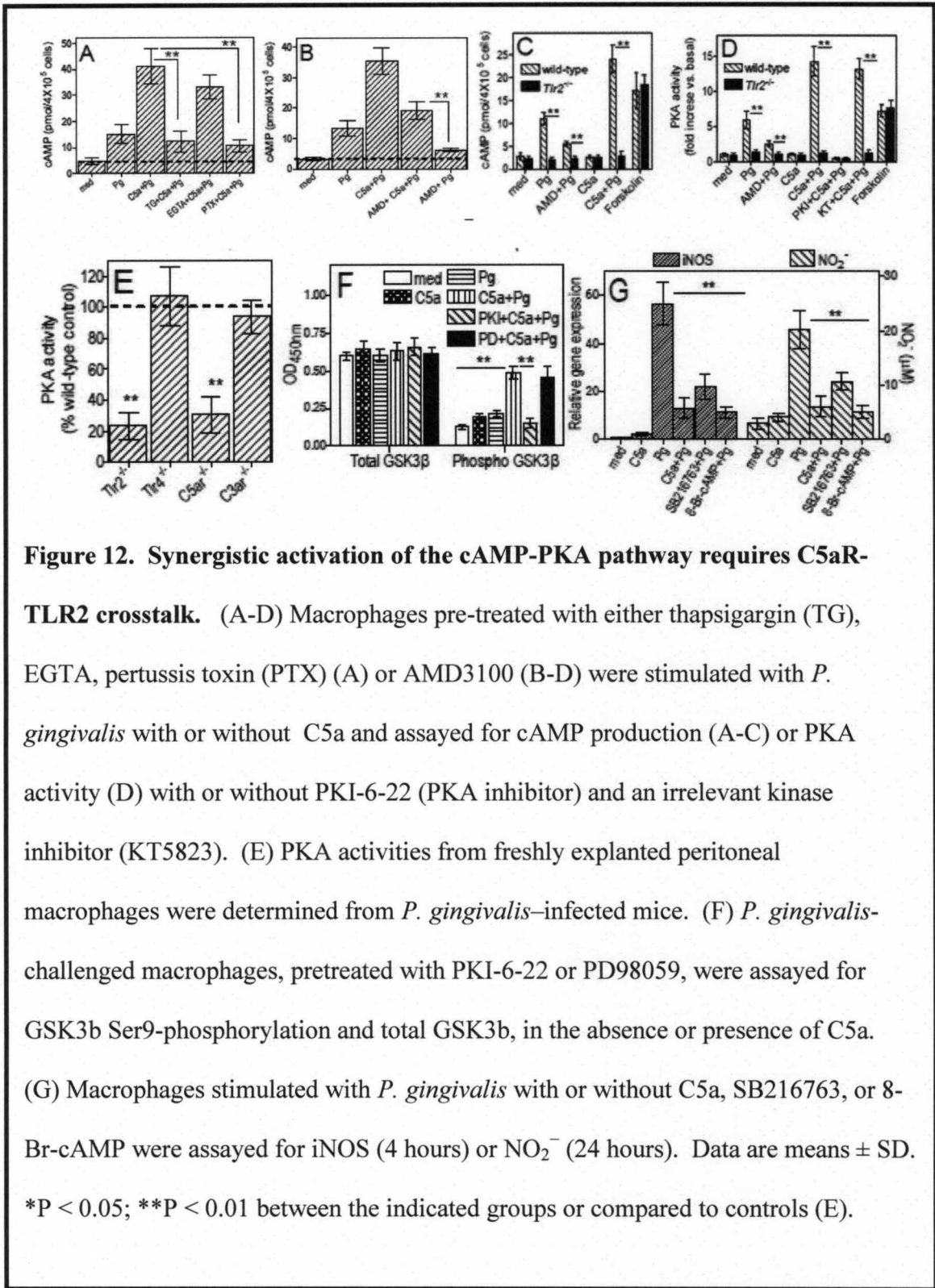
positive control) (Fig. 12G). Thus, GSK3 $\beta$  appears to regulate iNOS and nitric oxide downstream of PKA in C5a plus *P. gingivalis*-challenged macrophages.

The C5aR-TLR2 crosstalk is also consistent with confocal microscopy findings revealing for the first time co-localization of the two receptors in *P. gingivalis*-stimulated macrophages (Fig. 13B), and with fluorescence resonance energy transfer (FRET) experiments indicating that C5aR, TLR2, and *P. gingivalis* come into molecular proximity (Fig. 13A). Indeed, FRET analysis revealed significant energy transfer between Cy3-labeled C5aR and Cy5-labeled TLR2 in *P. gingivalis*-stimulated but not resting macrophages (Fig. 13A). No significant energy transfer was detected between Cy3-labeled C5aR and Cy5-labeled TLR5 or MHC Class I (controls) under the same conditions (Fig. 13A). Moreover, significant energy transfer was observed between FITC-labeled *P. gingivalis* and TRITC-labeled C5aR or TLR2 (but not TLR5 or MHC Class I) (Fig. 13A). However, unlike TLR2 which can directly be engaged by *P. gingivalis* (59) (137), C5aR appeared to associate indirectly with *P. gingivalis* in a TLR2-dependent way; indeed, the *P. gingivalis*-C5aR FRET association was abrogated in *Tlr2*<sup>-/-</sup> macrophages (Fig. 13A). Taken together, the findings firmly establish a crosstalk between C5aR and TLR2 for synergistic induction of cAMP signaling.

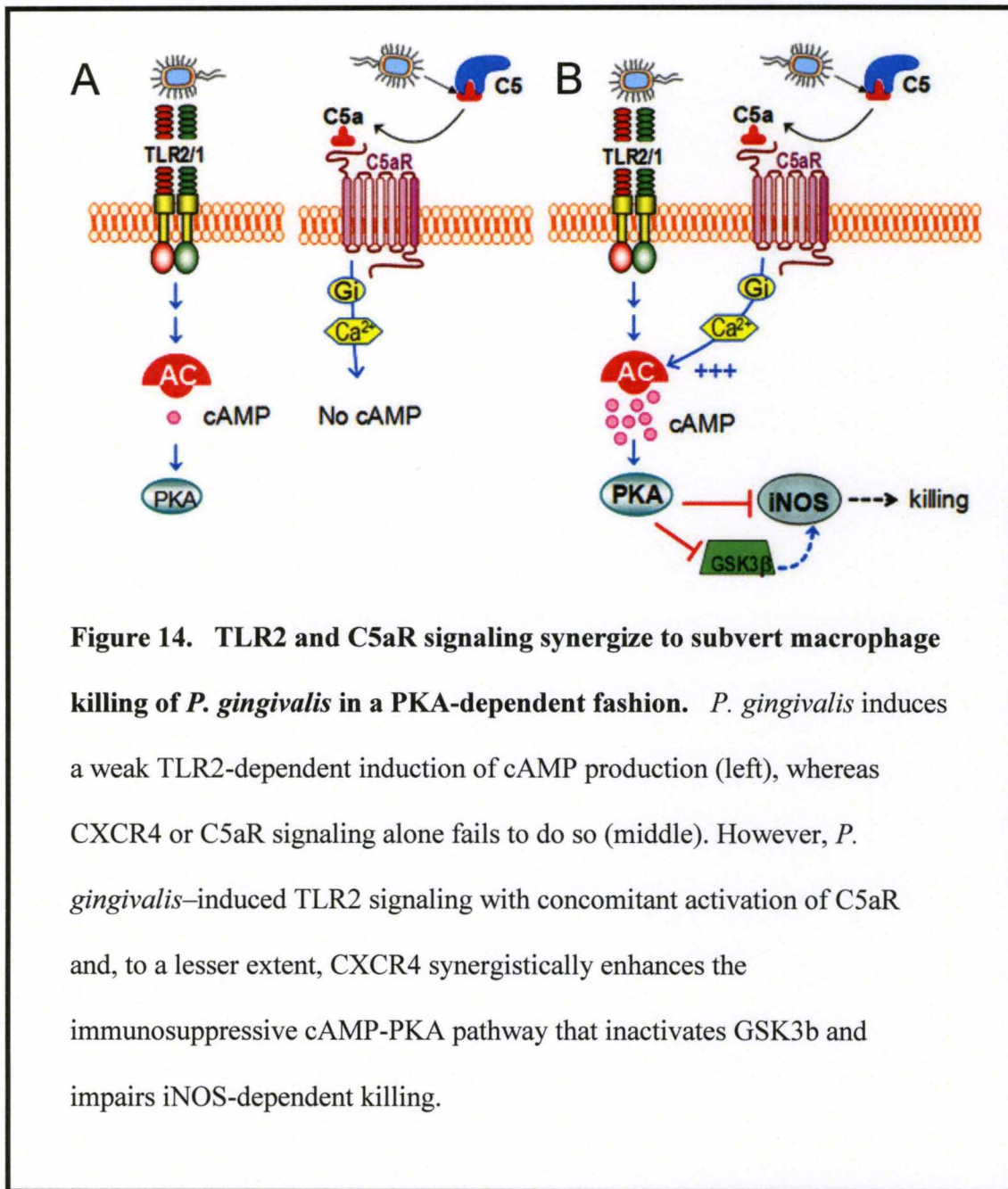
FRET analysis further revealed that in *P. gingivalis*-challenged macrophages, C5aR also associates with CXCR4 (Fig. 13A), suggesting co-association of all three receptors (CXCR4, TLR2, C5aR). These interactions likely occur in lipid rafts since all three receptors (but not TLR5 or MHC Class I) come within FRET proximity with an established lipid raft marker (GM1 ganglioside) in *P. gingivalis*-stimulated macrophages, unless the rafts are disrupted by methyl- $\beta$ -cyclodextrin (data not shown). Although the



C5aR-TLR2 crosstalk can proceed independently of CXCR4 and potently upregulates cAMP (Fig. 12B), maximal cAMP induction requires cooperation of all three receptors (Fig. 14).







**Figure 14. TLR2 and C5aR signaling synergize to subvert macrophage killing of *P. gingivalis* in a PKA-dependent fashion.** *P. gingivalis* induces a weak TLR2-dependent induction of cAMP production (left), whereas CXCR4 or C5aR signaling alone fails to do so (middle). However, *P. gingivalis*-induced TLR2 signaling with concomitant activation of C5aR and, to a lesser extent, CXCR4 synergistically enhances the immunosuppressive cAMP-PKA pathway that inactivates GSK3 $\beta$  and impairs iNOS-dependent killing.

## DISCUSSION

A molecular crosstalk between the complement system and the TLRs seems essential to appropriately coordinate the early innate response to infection (63) (165). Here, we addressed the intriguing possibility that at least some of the complement-TLR interplay may be instigated by pathogens, such as *P. gingivalis*, for promoting their adaptive fitness. The necessity for this evasion mechanism may be related to the fact that *P. gingivalis* cannot antagonize TLR2 activation at the receptor level, as it does with TLR4 (113). Therefore, it can be stated that this pathogen has evolved a subversive C5aR-TLR2 crosstalk mechanism for blunting the TLR2 antimicrobial response (Fig. 14), as an alternative to direct TLR2 antagonism. Notably, *P. gingivalis* does not rely on immunological mechanisms for C5aR activation, since it can activate this receptor through gingipain-mediated local generation of C5a (69). We confirmed and expanded the biochemical demonstration of C5a generation by purified gingipains acting on purified C5 substrate (69), by estimating that *P. gingivalis* generates high levels of C5a ( $32.7 \pm 4.3$  nM) upon 30-min incubation in heat-inactivated human serum. Notably, unlike C5a, C3a is extensively degraded and inactivated by *P. gingivalis* (69). Since C3a (but not C5a) exerts direct bactericidal effects (113), C3a destruction by *P. gingivalis* may serve to protect this pathogen.

The striking ability of C5a to synergize for cAMP production with *P. gingivalis* in a pertussis toxin-sensitive and TLR2-dependent way could be explained as follows. The Gi $\beta\gamma$  subunits, released upon activation of the pertussis toxin-sensitive Gai subunit, can potentially regulate adenylate cyclase (AC) activity, either positively or negatively depending on the enzyme isoform (107). Thus, although Gi $\beta\gamma$  cannot stimulate AC by

themselves, they can dramatically upregulate the activity of several AC isoforms in the presence of an appropriate stimulus. Such stimulus is apparently provided by *P. gingivalis* activation of TLR2. Importantly, the AC isoforms which are positively regulated by  $G_i\beta\gamma$  are not those that are sensitive to the inhibitory action of  $G_{\alpha i}$  (107). Since the ability of C5a to synergize with *P. gingivalis* for cAMP production is additionally dependent on intracellular  $Ca^{2+}$ ,  $G_i\beta\gamma$  may possibly mediate their stimulatory effects on AC activity through their  $Ca^{2+}$ -mobilizing effects.

A major mechanism underlying the regulatory effects of cAMP on cell activation involves the ability of cAMP-dependent PKA to phosphorylate the cAMP response element-binding protein (CREB), which effectively competes with the p65 subunit of nuclear factor- $\kappa$ B for limiting amounts of common transcriptional cofactors (125). Besides being under nuclear factor- $\kappa$ B control, the iNOS is additionally regulated by IFN- $\gamma$ ; interestingly, however, PKA also inhibits the IFN regulatory factor-1 that is required for the synergistic IFN- $\gamma$  contribution to iNOS transcription (24) (101). Moreover, as supported by the figure 12F data, PKA can phosphorylate and inactivate GSK3 $\beta$ , thus abrogating its stimulatory effect on pro-inflammatory gene expression (47). Since PKA activation causes greater iNOS inhibition than GSK3 $\beta$  inactivation (Fig. 12G), it is likely that PKA may inhibit iNOS also in a GSK3 $\beta$ -independent way (Fig. 14).

Although modest TLR-induced cAMP induction may control excessive pro-inflammatory signaling, sustained high levels of cAMP instigated by pathogens (and thus out of host control) may impair host defense. *P. gingivalis* is the first pathogen shown to exploit complement and TLRs to cause cAMP-dependent immune subversion *in vitro* and *in vivo*. It should be noted, however, that the interaction of C5a with *P. gingivalis*-

challenged macrophages did not induce a generalized or nonspecific macrophage immunosuppression, since C5a actually enhanced *P. gingivalis*-induced interleukin-6 (IL-6) production (data not shown). This sophisticated subversive crosstalk instigated by *P. gingivalis* (Fig. 14) serves in lieu of “built-in” adenylate cyclase which is not expressed by this bacterium, in contrast to *Bordetella pertussis* which disables human or mouse phagocytes by means of its own adenylate cyclase (14).

Macrophages can interact with *P. gingivalis* not only in periodontal tissues but also in the setting of systemic inflammatory diseases such as atherosclerosis (10) (114) (160). Our previous findings that *P. gingivalis* persists intracellularly in macrophages for at least 72h (147) were confirmed by an independent group, which additionally showed that up to 25% of the cells undergo necrosis by 72h and release cellular contents (100). It is thus conceivable that viable *P. gingivalis* could be released from necrotic macrophages, especially in the presence of C5a which dramatically promotes its intracellular persistence. This possibility becomes intriguing in view of epidemiological and mechanistic links between periodontitis and atherosclerosis (10) (114). However, whether the documented localization of viable *P. gingivalis* bacteria in atherosclerotic plaques (27) can be attributed to relocation of infected macrophages from periodontal tissues is currently uncertain. Nevertheless, the pathogen’s capacity to exit initially infected host cells and then enter and multiply within new hosts, including vascular cells, has been documented (29) (156).

C5aR activation in macrophages was also shown to inhibit TLR4-induced mRNA expression of IL-12p35, IL-12/IL-23p40, and IL-23p19, and production of IL-12p70 and IL-23 protein, through C5a-induced phosphatidylinositol-3 kinase and extracellular



signal-regulated kinase 1/2 signaling (63) (64) (165). The physiological significance of these C5a regulatory effects is likely to attenuate potential tissue damage mediated by various T cell effector subsets (*e.g.*, Th1 and Th17, regulated by IL-12 and IL-23, respectively), as seen in various pathological inflammatory conditions (91). However, undesirable outcomes may arise when C5a is not produced physiologically but rather through the uncontrolled action of microbial enzymes. In this context, pathogen-induced generation of C5a may modify TLR signaling and skew the T helper response in ways that could interfere with protective immunity. Therefore, on the basis of our findings and the reports on IL-12 and IL-23 regulation by C5a, it becomes evident that pathogens may exploit TLR-C5aR crosstalk in various ways.

In summary, this work constitutes the first report of complement-TLR crosstalk for synergistic cAMP induction which disables macrophages. From a therapeutic viewpoint, C5aR blockade effectively deprived this pathogen of crucial survival tactics and may thus confer protection against periodontitis and associated systemic diseases like atherosclerosis. Since C5a can be generated by both complement and non-complement C5 convertases that also include microbial enzymes (69) (112) (136), it becomes important to identify other pathogens that exploit C5a-mediated subversive crosstalk signaling with TLRs. This will have important implications for novel counter-strategies to neutralize microbial virulence. Our findings further suggest that, in the course of evolution, chronically persisting pathogens may not have simply “learned” to breach complement and the TLRs separately, but, as hereby exemplified by *P. gingivalis*, to also exploit their communication hubs.



## CHAPTER FIVE: THE C5A RECEPTOR IMPAIRS IL-12-DEPENDENT CLEARANCE OF PORPHYROMONAS GINGIVALIS AND IS REQUIRED FOR INDUCTION OF PERIODONTAL BONE

### Introduction

In addition to its role in pathogen recognition and elimination, the complement network regulates immune and inflammatory responses through direct effects on immune cells or via crosstalk with TLRs and other signaling pathways (130). Both complement and TLRs are rapidly activated in response to infection and their crosstalk may serve to coordinate the host response through synergistic or antagonistic interactions. These interactions may respectively enhance host defense or control it to prevent immunopathology. However, the propensity of complement and TLRs for communication may be exploited by microbial pathogens to manipulate the host response in ways that promote their adaptive fitness (52).

In this context, we have recently shown that the periodontal pathogen *Porphyromonas gingivalis* induces a subversive crosstalk between the complement C5a receptor (C5aR) and TLR2 that impairs nitric oxide-dependent intracellular killing in macrophages (157). Interestingly, *P. gingivalis* can control both receptors: it can directly engage TLR2 through cell-surface ligands (4), whereas it can activate C5aR (CD88) through local conversion of C5 to C5a using its own enzymes (157). Indeed, this bacterium does not have to rely on immunological mechanisms for C5a generation, but

rather expresses extracellular cysteine proteinases (gingipains) which function as C5 convertase-like enzymes (125) (157).

C5aR activation has been also shown to downregulate TLR4-induced production of IL-12 *in vitro* and *in vivo* (63) (85) (165). This effect is exerted at the transcriptional level since C5aR signaling in macrophages inhibits TLR4-induced mRNA expression of the IL-12p35 and IL-12/IL-23p40 subunits. Since IL-12 is a key cytokine in Th1 differentiation and cell-mediated immunity, this C5aR-TLR4 crosstalk may represent a regulatory mechanism to control IL-12 production and thereby prevent or attenuate possible immunopathology (52). However, undesirable outcomes could arise if C5a is produced at excessively high levels, as may happen in sepsis. Under such conditions, the crosstalk between C5a-activated C5aR and TLR4 could severely suppress IL-12 and interfere with protective Th1 immunity (52) (63).

High levels of C5a can be generated also through the uncontrolled action of C5-convertase-like microbial enzymes like the *P. gingivalis* gingipains (157). We therefore hypothesized that *P. gingivalis* may take advantage of C5a-induced signaling to suppress biologically active IL-12 (IL-12p70). Given that IL-12p70 induces IFN- $\gamma$  and mediates bacterial clearance through activated phagocytes (153), possible inhibition of this cytokine by *P. gingivalis* through C5aR exploitation could contribute to its ability to evade immune control. In this chapter, we show that C5a (and, to a lesser extent, its desarginated derivative C5a<sup>desArg</sup>) inhibits TLR2-dependent induction of IL-12p70, but enhances induction of pro-inflammatory and bone-resorptive cytokines (IL-1 $\beta$ , IL-6, and TNF- $\alpha$ ), in response to *P. gingivalis*. These *in vitro* observations were confirmed by *in vivo* studies, which additionally showed that C5aR-dependent inhibition of IL-12p70

promotes the survival of this pathogen. Moreover, C5aR signaling was required for the ability of *P. gingivalis* to induce periodontal bone loss in a mouse model of experimental periodontitis. Therefore, *P. gingivalis* exploits C5aR to promote its adaptive fitness and cause periodontal disease. This immune subversion mechanism has important therapeutic implications given the current availability of safe, selective, and potent C5aR antagonists.

## Results

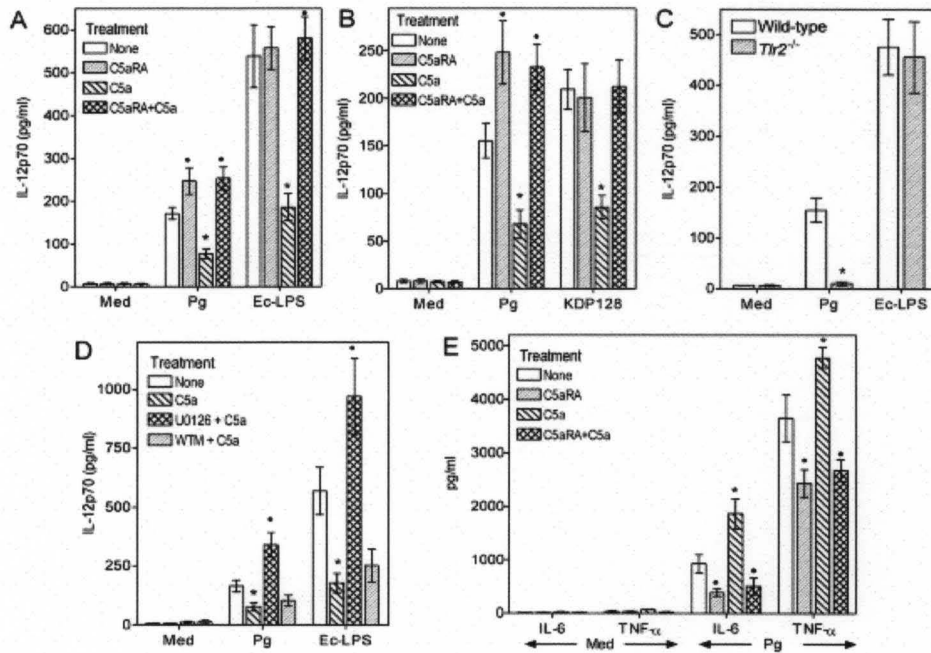
### ***P. gingivalis* proactively and selectively inhibits IL-12p70 production via C5aR-TLR2 crosstalk**

We investigated whether C5a inhibits *P. gingivalis*-induced IL-12p70 in peritoneal macrophages. *E. coli* LPS-stimulated macrophages were used as a control since C5a has been shown to inhibit IL-12p70 through a C5a/C5aR–LPS/TLR4 crosstalk (63). The host TLR response against *P. gingivalis* is predominantly mediated by TLR2 both *in vitro* and *in vivo* (54) (55) (11). Therefore, we additionally examined whether possible C5a-mediated inhibition of *P. gingivalis*-induced IL-12p70 could involve a C5aR-TLR2 crosstalk. We found that the abilities of both *P. gingivalis* and LPS to induce IL-12p70 production were significantly inhibited by C5a ( $p < 0.01$ ; Fig. 15A). These inhibitory effects were specifically mediated by C5aR signaling since they were completely reversed by a specific C5aR antagonist (C5aRA) ( $p < 0.01$ ; Fig. 15A).

Intriguingly, we observed that C5aR blockade significantly enhanced the induction of IL-12p70 production, even in *P. gingivalis*-stimulated macrophages that were not treated with exogenous C5a ( $p < 0.01$ ; Fig. 15A). However, we did not observe this upregulatory effect of C5aR antagonism in LPS-stimulated macrophages (Fig. 15A).

We previously showed that *P. gingivalis* uses its gingipains to generate C5a in complement-inactivated serum (157). Therefore, we reasoned that endogenously generated C5a limits *P. gingivalis*-induced IL-12p70 production, which is thus enhanced in the presence of C5aRA. This notion was substantiated by the finding that the isogenic mutant KDP128, which lacks all three gingipain genes, failed to regulate IL-12p70, unless exogenous C5a was added in the cell cultures (Fig. 15B). Indeed, C5aRA had no effect on KDP128-induced IL-12p70 in the absence of exogenously added C5a (Fig. 15B). The ability of *P. gingivalis* to induce IL-12p70 was completely abrogated in TLR2-deficient macrophages, whereas, as expected, LPS-induced IL-12p70 was unaffected (Fig. 15C). Taken together, these data indicate that *P. gingivalis* activates a C5aR-TLR2 crosstalk which inhibits IL-12p70 production in macrophages.

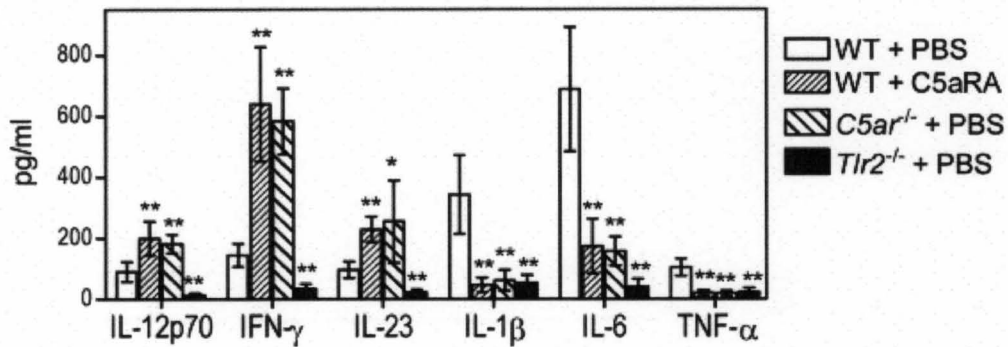
The C5aR crosstalk pathways with TLR2 or TLR4 for IL-12p70 regulation appear to be similar, since the inhibitory effects of C5a were abrogated by treatment with the MEK1/2-specific inhibitor U0126 but not by the PI3K inhibitor wortmannin ( $p < 0.01$ ; Fig. 15D). This implicates the MEK-ERK1/2 pathway in C5aR-mediated regulation of both TLR2- and TLR4-induced IL-12p70. On the other hand, the PI3K pathway is minimally involved, if at all (63). The C5aR-dependent inhibition of IL-12p70 in *P. gingivalis*-stimulated macrophages was selective for this cytokine, since other pro-inflammatory cytokines (IL-6 and TNF- $\alpha$ ) were augmented ( $p < 0.01$ ; Fig. 1E). In conclusion, *P. gingivalis* proactively and selectively inhibits IL-12p70 production by activating a C5aR-TLR2 crosstalk without requirement for immunological mechanisms of complement activation.



**Figure 15: C5aR signaling inhibits TLR2-dependent IL-12p70 induction in *P. gingivalis*-activated macrophages.** IFN- $\gamma$  primed wild-type (A-D) or TLR2-deficient (C) mouse peritoneal macrophages were stimulated with medium, *P. gingivalis*, or *E. coli* LPS. Panel B includes the use of an isogenic mutant (KDP128) which is deficient in all three gingipain genes. Macrophages were pre-treated with C5aRA (A-B), U0126 or wortmannin (D) prior to challenge with C5a, *P. gingivalis*, or Ec-LPS. Culture supernatants were assayed for induction of the indicated cytokines after 24h of incubation. Data are means  $\pm$  SD ( $n = 3$  sets of macrophages). Asterisks show statistically significant ( $p < 0.01$ ) inhibition (A-D; IL-12p70) or enhancement (E; IL-6 and TNF- $\alpha$ ) of cytokine production, whereas black circles indicate statistically significant ( $p < 0.01$ ) reversal of these modulatory effects.

## **C5aR signaling *in vivo* differentially regulates *P. gingivalis*-induced cytokine responses**

We next investigated the biological significance of the C5aR-mediated inhibition of IL-12p70 production. First, it was essential to determine whether C5aR signaling can regulate *P. gingivalis*-induced IL-12p70 production also *in vivo*. For this purpose, wild-type mice were i.p. administered C5aRA followed by i.p. challenge with *P. gingivalis*. Mice deficient in C5aR or TLR2 were similarly challenged with *P. gingivalis*, and all mice were sampled 5h post-infection by peritoneal lavage. In addition to IL-12p70, we determined production of IFN- $\gamma$  (which is positively regulated by IL-12p70 (153)), IL-23 (an IL-12 family cytokine which shares a common IL-12/IL-23p40 subunit with IL-12p70 (119)), as well as pro-inflammatory cytokines that have been implicated in inflammatory bone resorption in periodontitis (IL-1 $\beta$ , IL-6, and TNF- $\alpha$ ) (43). C5aRA-treated wild-type mice and C5aR-deficient mice elicited significantly higher levels of IL-12p70, IFN- $\gamma$ , and IL-23 compared to PBS-treated wild-type controls ( $p < 0.01-0.05$ ; Fig. 16). In contrast, the induction of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  production was inhibited by C5aR blockade or C5aR deficiency ( $p < 0.01$ ; Fig. 2). On the other hand, the induction of all tested cytokines was abrogated in TLR2-deficient mice ( $p < 0.01$ ; Fig. 2). These data confirm that C5aR signaling *in vivo* selectively inhibits the ability of *P. gingivalis* to induce TLR2-dependent IL-12 family cytokines (IL-12p70 and IL-23). Additionally, the observed downregulation of IFN- $\gamma$  is most likely secondary to inhibition of IL-12p70 production. On the other hand, maximal induction of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  requires intact signaling by both C5aR and TLR2.

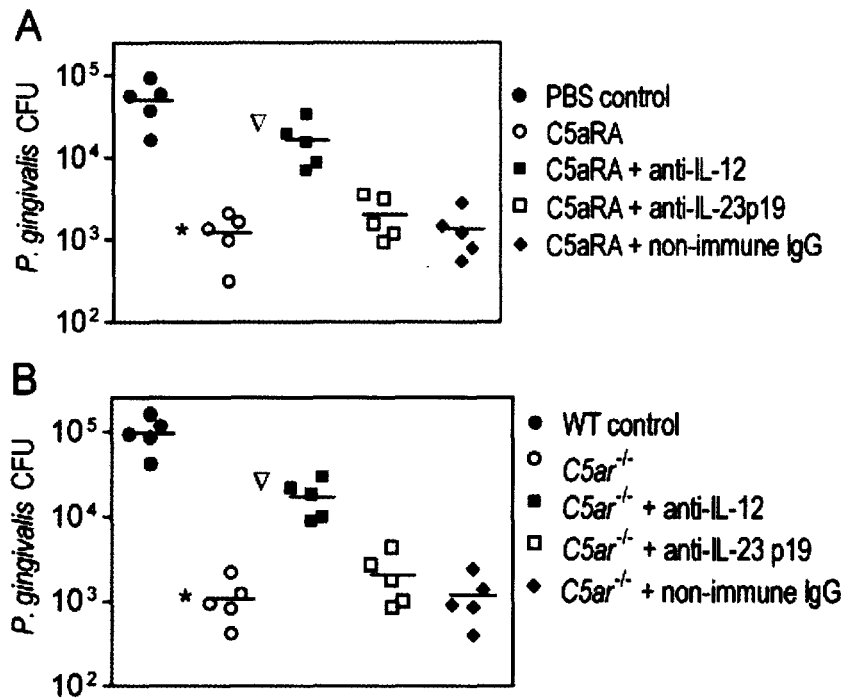


**Figure 16. C5aR signaling regulates *P. gingivalis*-induced and TLR2-dependent cytokine production in vivo.** 10-12 week-old wild-type (WT) mice, which were pretreated or not with C5aRA (i.p.; 25  $\mu$ g/mouse), as well as mice deficient in C5aR (*C5ar*<sup>-/-</sup>) or TLR2 (*Tlr2*<sup>-/-</sup>), were i.p. infected with *P. gingivalis* ( $5 \times 10^7$  CFU). Peritoneal lavage was performed 5h post-infection and the peritoneal fluid was used to measure the levels of the indicated cytokines. Mice not infected with *P. gingivalis* had undetectable levels of the cytokines investigated. Data are means  $\pm$  SD ( $n = 5$  mice). \*,  $p < 0.01$  and \*\*,  $p < 0.01$  vs. WT+PBS control.

### **C5aR-mediated inhibition of IL-12p70 promotes *P. gingivalis* survival *in vivo***

Whether the C5aR-mediated inhibitory effect on IL-12p70 production (Fig. 16) is exploited by *P. gingivalis* was addressed in subsequent experiments. Wild-type mice were i.p. treated with C5aRA (or PBS control) and infected with *P. gingivalis* by the same route. The C5aRA-treated mice comprised several groups, including mice given anti-IL-12 IgG, anti-IL-23p19 IgG, or non-immune IgG control. Treatment with anti-IL-23p19 was included because the anti-IL-12 Ab reacts with both IL-12p70 subunits, p35 and p40, the latter of which is shared by the heterodimeric IL-23 (IL-12/IL-23p40 and IL-23p19 (119)). Thus, the experiment was designed in a way that would allow specific implication of IL-12p70 or both IL-12p70 and IL-23 in *P. gingivalis* immune clearance. At 24h post-infection, the peritoneal lavage fluid from C5aRA-treated mice contained about 2 log<sub>10</sub> units less *P. gingivalis* CFU compared to mice pretreated with PBS control ( $p < 0.01$ ; Fig. 17A). However, the enhanced ability of C5aRA-treated mice to clear *P. gingivalis* was significantly ( $p < 0.01$ ) counteracted by anti-IL-12 treatment, though not by anti-IL-23p19 or non-immune IgG (Fig. 17A). Viable *P. gingivalis* CFU counts were not detected in the blood or in homogenates of several organs examined (spleen, kidney, liver, and lungs) from any of the mouse groups. Taken together with the Fig. 16 findings, these data show that C5aR signaling inhibits IL-12p70 production and this inhibitory effect is exploited by *P. gingivalis* to resist immune clearance. This conclusion was further substantiated by similar findings from a related experiment in which C5aRA-treated mice were replaced by C5aR-deficient mice (Fig. 17B).





**Figure 17. Inhibition of C5aR signaling promotes the *in vivo* clearance of *P. gingivalis* by augmenting IL-12.** (A) Wild-type mice were pre-treated or not with C5aRA (i.p.; 25  $\mu$ g/mouse), in the presence or absence of goat polyclonal anti-mouse IL-12 IgG, anti-mouse IL-23p19 IgG, or equal amount of non-immune IgG (i.p.; 0.1 mg/mouse). The mice were then infected i.p. with *P. gingivalis* ( $5 \times 10^7$  CFU). (B) Similar experiment in which C5aRA-treated mice were replaced by C5aR-deficient (*C5ar*<sup>-/-</sup>) mice. Peritoneal lavage was performed 24h post-infection and the peritoneal fluid was used to determine viable *P. gingivalis* CFU counts. Data are shown for each individual mouse with horizontal lines indicating mean values. \*,  $p < 0.01$  vs. controls. The inverted triangles indicate significant ( $p < 0.01$ ) reversal of the effects of C5aRA or C5aR deficiency by anti-IL-12.

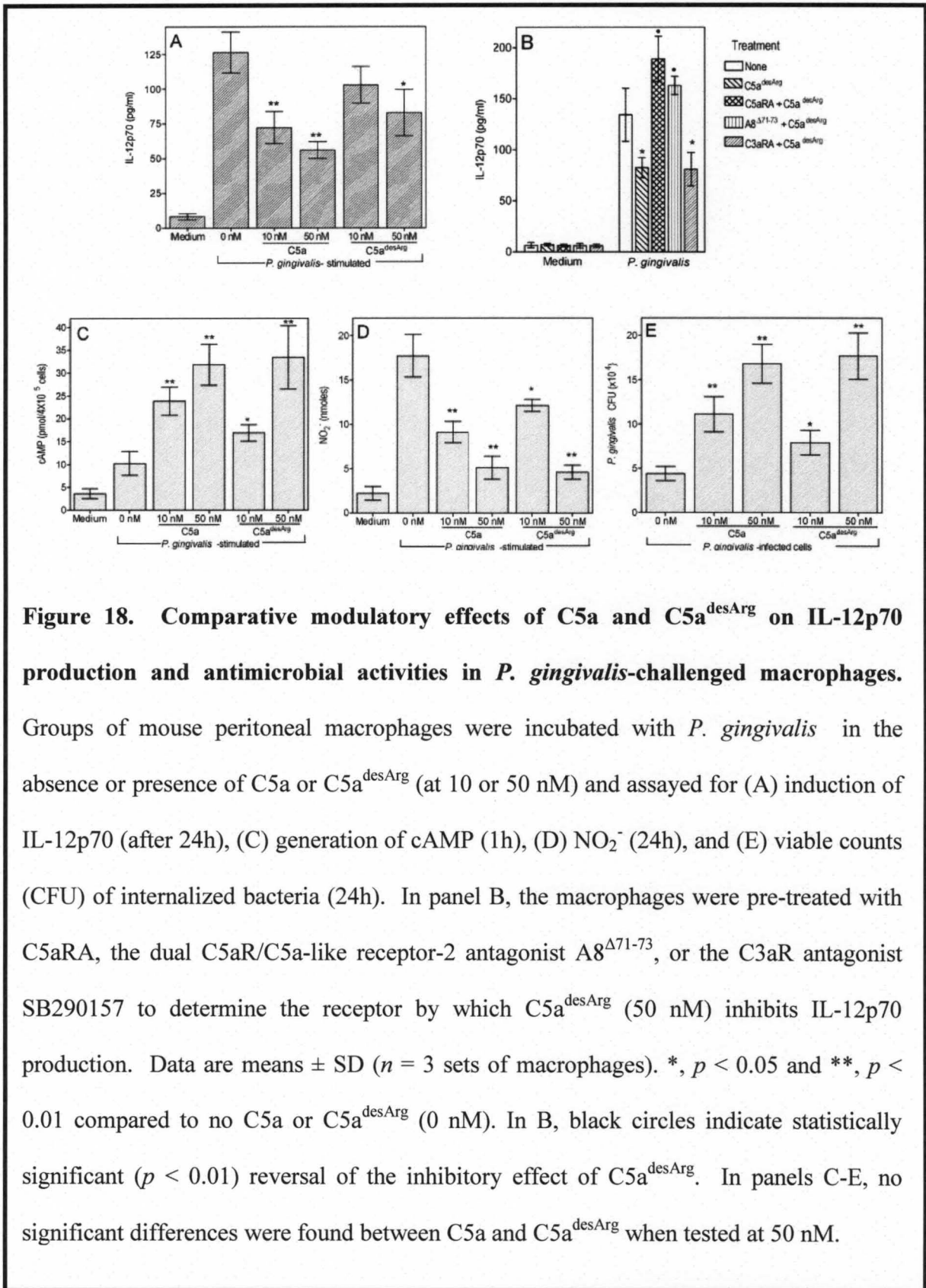
## **Comparison of C5a and C5a<sup>desArg</sup> in regulating IL-12p70 and other macrophage activities**

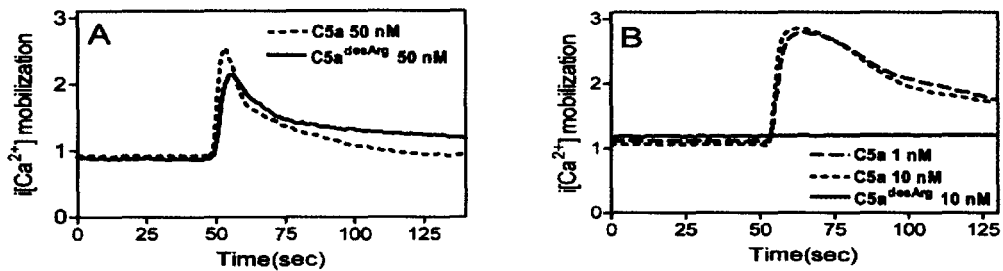
The peritoneal fluid of *P. gingivalis*-infected mice from the above described experiments was also assayed by mouse C5a ELISA. C5a was detected at  $2304 \pm 527$  pg/ml ( $n = 5$  mice) and  $1629 \pm 378$  pg/ml ( $n = 5$  mice) at, respectively, 5h and 24h post-infection. C5a is relatively unstable in biological fluids and is rapidly converted to its desarginated form (C5a<sup>desArg</sup>). In fact, a large part of detected C5a may represent C5a<sup>desArg</sup> since the capturing antibody used in the sandwich ELISA (BD Pharmingen) recognizes a neoepitope exposed in both C5a and C5a<sup>desArg</sup> (though not in intact C5). C5a<sup>desArg</sup> does not have anaphylactic action but retains a number of other biological activities (97) (102) (107). We thus investigated whether it shares the capacity of C5a to regulate IL-12p70. We found that C5a<sup>desArg</sup> can also inhibit *P. gingivalis*-induced IL-12p70 production, though not as strongly as C5a. Specifically, C5a<sup>desArg</sup> mediated significant ( $p < 0.05$ ) inhibition of IL-12p70 at 50 nM but not at 10 nM, at which concentration C5a was already effective (Fig. 18A). However, the increased stability and thus higher prevalence of C5a<sup>desArg</sup> compared to intact C5a suggests a possible significant role for the desarginated molecule in IL-12p70 regulation.

Although C5a<sup>desArg</sup> binds also to the C5a-like receptor-2 (C5L2) with high affinity (120) (130), its observed modulatory effect on IL-12p70 production was likely mediated via the C5aR (CD88). In this regard, C5aRA by itself caused full reversal of the inhibitory effect of C5a<sup>desArg</sup>, whereas a dual C5aR/C5a-like receptor-2 antagonist (A8<sup>Δ71-73</sup>) (120) (133) had a comparable effect (Fig. 18B). In contrast, the C3aR antagonist

SB290157 (control) did not influence the ability of C5a<sup>desArg</sup> to inhibit induction of IL-12p70 by *P. gingivalis* (Fig. 18B).

We previously implicated C5a in synergistic interactions with *P. gingivalis* that elevate cAMP in macrophages, leading to inhibition of nitric oxide production and of intracellular killing (157). We investigated whether these evasion mechanisms can also be activated by C5a<sup>desArg</sup>. Side-by-side comparison revealed no significant differences between C5a and C5a<sup>desArg</sup> when tested at 50 nM in elevating cAMP, inhibiting nitric oxide, and promoting its intracellular survival (Fig. 18, C-E). However, when the compounds were tested at 10 nM, C5a exhibited stronger effects than C5a<sup>desArg</sup> (Fig. 18, C-E). In view of the strict dependence of C5a on intracellular Ca<sup>2+</sup> mobilization to synergistically elevate cAMP (157), we hypothesized that C5a<sup>desArg</sup> could similarly induce intracellular Ca<sup>2+</sup> responses. Indeed, at 50 nM, C5a and C5a<sup>desArg</sup> induced comparable intracellular Ca<sup>2+</sup> mobilization in macrophages (Fig. 19A), whereas only C5a was active in that regard in neutrophils (Fig. 19B). Taken together, the data from Figs. 18 and 19 indicate that *P. gingivalis* can exploit C5a even after its conversion to C5a<sup>desArg</sup>, thereby undermining macrophage defense functions (induction of IL-12p70, activation of intracellular killing).



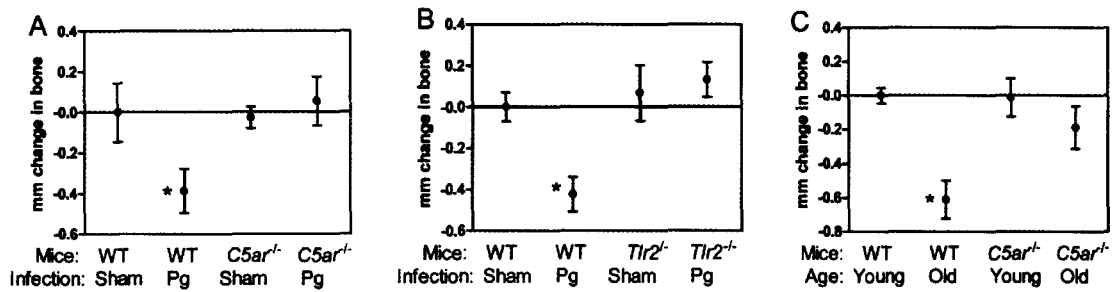


**Figure 19. Comparison of C5a and C5a<sup>desArg</sup> in intracellular Ca<sup>2+</sup> mobilization.** Mouse peritoneal macrophages (A) or neutrophils (B) were loaded with the ratiometric calcium indicator Indo-1 AM and stimulated with C5a or C5a<sup>desArg</sup> at the indicated concentrations (lower concentrations were used for neutrophils since they are more sensitive to C5a than macrophages (49)). Ca<sup>2+</sup> mobilization was measured in a spectrofluorometer and the traces are representative of three experiments.

### **C5aR mediates periodontal bone loss**

The involvement of C5aR signaling in *P. gingivalis* immune evasion and in the induction of pro-inflammatory cytokines (Figs. 15-18), such as IL-1 $\beta$ , IL-6, and TNF- $\alpha$  that mediate periodontal bone resorption (43), suggested that C5aR may play an important role in *P. gingivalis*-induced periodontitis. Indeed, *P. gingivalis* failed to induce significant periodontal bone loss in C5aR-deficient BALB/c or C57BL/6 mice, in stark contrast to corresponding wild-type mice which developed significant bone loss relative to sham-infected controls ( $p < 0.01$ ; Fig. 20 A, B, and E). TLR2 participates in crosstalk interactions with C5aR that promote mechanisms of *P. gingivalis* immune evasion (157) and induce production of bone-resorptive cytokines (Fig. 16). Sensibly, therefore, TLR2-deficient BALB/c mice were similarly shown to be resistant to *P. gingivalis*-induced periodontal bone loss (Fig. 20 C and E).

Mice used for *P. gingivalis*-induced periodontitis studies are usually 8-12 week-old and sham-infected controls do not develop appreciable bone loss (44). However, aging mice, like aging humans, gradually develop naturally-occurring inflammatory periodontal bone loss (due to chronic exposure to indigenous periodontal bacteria), which becomes quite dramatic after 9 months of age (51) (93). To determine the role of C5aR in the age-associated periodontitis model, we raised C5aR-deficient BALB/c mice and wild-type controls until the age of 16 months. We found that old C5aR-deficient mice are significantly protected against age-associated periodontitis relative to similarly aged wild-type controls ( $p < 0.01$ ; Fig. 20D). Therefore, C5aR is involved in chronic, age-associated periodontal bone loss. However, it is currently uncertain whether C5aR is exploited by mouse periodontal bacteria as shown for *P. gingivalis*.



**Figure 20. C5aR and TLR2 deficiencies protect against periodontal bone loss.**

Mice deficient in C5aR (*C5ar*<sup>-/-</sup>) (A, BALB/c; B, C57BL/6) or TLR2 (*Tlr2*<sup>-/-</sup>) (C; BALB/c) and appropriate wild-type controls were orally infected or not with *P. gingivalis* and assessed for induction of periodontal bone loss six weeks later. Mice used in these experiments were 10-12 week-old. (D) Induction of naturally occurring periodontal bone loss in 16-month-old wild-type or *C5ar*<sup>-/-</sup> BALB/c mice relative to their young counterparts ( $\leq 12$  weeks of age). (E) Representative images of *P. gingivalis*-induced bone loss under wild-type or C5aR- or TLR2-deficient conditions: *P. gingivalis*-infected *C5ar*<sup>-/-</sup> or *Tlr2*<sup>-/-</sup> mice display considerably smaller CEJ-ABC distances (yellow arrows) compared to infected wild-type mice, but quite comparable to those of sham-infected wild-type mice. Data are means  $\pm$  SD ( $n = 5$  mice). \*,  $p < 0.01$  compared to corresponding sham-infected controls (A and B) or young counterparts (C).

## Discussion

Clinical and histological observations implicate complement in periodontal inflammation and pathogenesis, although the precise mechanisms or pathways involved have remained largely undefined (48). However, our findings clearly implicate the C5a-C5aR axis in periodontal tissue destruction and immune evasion by periodontal bacteria.

Our present data suggest that *P. gingivalis* may exploit C5aR to promote its adaptive fitness in diverse ways. On the one hand, C5aR signaling inhibits TLR2-dependent IL-12p70 induction and interferes with immune clearance of *P. gingivalis*. On the other hand, the *P. gingivalis*-instigated C5aR-TLR2 crosstalk leads to enhanced production of other pro-inflammatory cytokines (IL-1 $\beta$ , IL-6, and TNF- $\alpha$ ). Therefore, this pathogen does not appear to cause a generalized immunosuppression but, rather, has evolved the ability to selectively target pathways that could result in its elimination. Consequently, *P. gingivalis* is an asaccharolytic organism with a strict requirement for peptides and heme, and thus depends on the continuous flow of inflammatory serum exudate (gingival crevicular fluid) to obtain these essential nutrients and survive in its periodontal niche (34). In fact, non-selective immunosuppression would not be advantageous for *P. gingivalis*; whereas such strategy would certainly afford protection against host immunity; however, *P. gingivalis* would likely be condemned to starvation. . Therefore, the proactive release of C5a by *P. gingivalis* and the ensuing C5a-induced inflammation (increased vascular permeability and pro-inflammatory synergy with TLRs) can contribute to nutrient procurement. Moreover, the ability of *P. gingivalis* to induce C5aR-dependent periodontal bone loss expands the useful space for increased niche for the pathogen.



On the basis of the above discussion, it becomes apparent that *P. gingivalis* uses a quite antithetical strategy relative to *Staphylococcus aureus* which promotes its survival by actually blocking C5a binding and C5aR activation, via a secreted protein known as CHIPS (chemotaxis inhibitory protein of *S. aureus*) (25). This mechanism inhibits C5a-induced inflammation and phagocytic cell chemotaxis and protects *S. aureus* from neutrophils and macrophages (25). On the other hand, the protozoan parasite *Leishmania major* also exploits C5aR for evading host immunity, which is restored in C5aR-deficient mice that consequently do not develop necrotizing dermal lesions as wild-type animals do (63). However, unlike *P. gingivalis*, *L. major* has to rely on C5a generation by the physiological complement cascade in order to exploit C5aR.

*P. gingivalis*-induced inflammation via the C5aR-TLR2 crosstalk may have important implications from a clinical perspective, since it is likely to cause collateral tissue damage (inflammatory periodontal bone destruction). This notion is supported by our findings that mice deficient in C5aR or TLR2 are both resistant to *P. gingivalis*-induced periodontitis. The fact that induction of bone loss is essentially prevented in the absence of either C5aR or TLR2 signaling, argues against the possibility that C5aR and TLR2 contribute to periodontal pathogenesis through independent effector mechanisms. In this regard, both receptors are under *P. gingivalis* control and are induced to crosstalk, while in physical proximity (157), cooperatively leading to immune evasion and induction of inflammatory/bone-resorptive cytokines.

Both the C5a and C3a anaphylatoxins are readily metabolized in serum and lose their C-terminal arginine due to carboxypeptidase activity (107). The resulting C3a fragment (C3a<sup>desArg</sup>) is biologically inert in terms of C3a receptor-dependent functions,

but retains antimicrobial activity which is exerted independently of the receptor (114). On the other hand, C5a<sup>desArg</sup> can still bind C5aR, albeit with a lower affinity and a different mode of interaction relative to intact C5a (19) (107). Although C5a<sup>desArg</sup> is devoid of C5a spasmogenic (anaphylactic) activity, it retains other C5a activities to varying degrees depending on function and cell type involved. For example, monocytes/macrophages, but not neutrophils, do not appear to distinguish between C5a and C5a<sup>desArg</sup> in terms of induction of chemotaxis or lysosomal enzyme release (13) (97) (102). Our findings that C5a<sup>desArg</sup> retains the ability to inhibit *P. gingivalis*-induced IL-12p70 and nitric oxide production has important implications: being considerably more stable than C5a (107), C5a<sup>desArg</sup> may provide a persisting stimulus for sustained manipulation of the antimicrobial response and destructive inflammation, properties that characterize chronic conditions like periodontitis. Intriguingly, whereas *P. gingivalis* attacks C5 and generates biologically active C5a/C5a<sup>desArg</sup>, it extensively degrades C3 and C3a which thus do not retain biological activity (161). Since C3a (but not C5a) exerts direct antimicrobial effects and readily kills both gram-negative and gram-positive bacteria (114), it is possible that degradation and inactivation of C3a by *P. gingivalis* may serve to protect this pathogen.

The data from this study collectively suggest that *P. gingivalis* has evolved to not only endure the host response (by selectively suppressing critical ‘killing’ pathways, such as IL-12–dependent clearance), but also to benefit from the inflammatory response, while at the same time contributing to periodontal pathogenesis. The ability of *P. gingivalis* to inhibit innate immune functions via C5aR exploitation may also allow bystander bacteria, *i.e.*, co-habiting the same niche, to evade immune control. In this context, *P. gingivalis* is

thought of as a keystone periodontal species that could promote the survival and virulence of the entire microbial community (20) (22) (49) (82).

In addition to being a prevalent and costly chronic condition that destroys tooth-supporting tissues, severe periodontitis exerts a systemic impact on health and the patients run increased risk for diseases such as atherosclerosis, diabetes, and perhaps rheumatoid arthritis (26) (30) (123) (151). Therefore, it becomes important to identify promising therapeutics for the treatment of this oral disease. Since C5aR- or TLR2-deficient mice are both resistant to periodontal bone loss, at least in principle, pharmacological blockade of either C5aR or TLR2 could inhibit periodontitis. However, the availability of highly effective and safe C5aR antagonists, some of which have completed phase II trials (for rheumatoid arthritis and psoriasis) (131) (155), and the relative paucity of effective TLR2 antagonists, suggest that C5aR is a preferential and promising target of local therapeutic intervention to treat human periodontitis. From a mechanistic viewpoint, C5aR blockade may counteract the ability of periodontal bacteria to evade critical antimicrobial responses or to stimulate non-resolving/destructive inflammation, and thus should be capable of both controlling the infection and inhibiting periodontal bone loss.

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## **CURRICULUM VITAE**

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Bachelor of Arts in Biology: University of Louisville, 5/2003

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Ph.D candidate: University of Louisville, 2004-present

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### **Peer-reviewed publications:**

**Jennifer L. Krauss**, Jan Potempa, John D. Lambris, and George Hajishengallis. Complementary Tolls in the periodontium: how periodontal bacteria modify complement and Toll-like receptor responses to prevail in the host. *Periodontology 2000*, vol.52, 2010, 141-162.

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inflammation. *Periodontal Disease: Methods and Protocols, Methods in Molecular Biology Series* 2010.

**Jennifer L. Krauss**, Shuang Liang, Megan L. McIntosh, and George Hajishengallis. The C5a receptor impairs IL-12-dependent clearance of *Porphyromonas gingivalis* and is required for induction of periodontal bone loss. *Manuscript in revision. Journal of Immunology*, 2010.

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**Presented Abstracts:**

***Pre-doctoral Research:***

**Jennifer L. Krauss**, Charla Mutchler, Josh B. Lane, Jason Hudkins, Jessica Miles and Rey A. Carabeo. Identification of two distinct, but functionally redundant pathways of Chlamydial invasion. Presented at Research Louisville, Louisville, KY. 10/2005.

**Jennifer L. Krauss** and Rey A. Carabeo. Growth characteristics of *Chlamydia trachomatis* in human monocyte-derived dendritic cells. Presented at the 3<sup>rd</sup> Biennial Meeting of the Chlamydia Basic Science Research Society, Louisville, KY. 03/2007.

**Jennifer L. Krauss** and Rey A. Carabeo. Fate of *Chlamydia trachomatis* in human phagocytic cells. Presented at the 14<sup>th</sup> Annual Midwest Microbial Pathogenesis Meeting, Chicago, IL. 09/2007.

**Jennifer L. Krauss**, Min Wang, Shuang Liang, John D. Lambris and George Hajishengallis. Manipulation of the complement system by *Porphyromonas gingivalis*. Presented at the Gordon Research Conference on Periodontal Disease. New Haven, New Hampshire. 08/ 2009.

**Jennifer L. Krauss**, Kathy Triantafilou, John D. Lambris and George Hajishengallis. 'Complementary' Immune Evasion Strategies of *Porphyromonas gingivalis*. Presented at the International Complement Workshop XXIII, New York, NY. 08/ 2010.

**Jennifer L. Krauss**, Kathy Triantafilou, John D. Lambris and George Hajishengallis. 'Complementary' Immune Evasion Oral Pathogen *Porphyromonas gingivalis*. Presented at Research Louisville, Louisville, KY. 10/2010.

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**Presented Seminar Lectures:**

***Pre-doctoral Research:***

The Role of Human Dendritic Cells in Chlamydial Pathogenesis. Presented to the University of Louisville Department of Microbiology and Immunology. 11/2006

*P. gingivalis*-induced TLR/Complement Receptor Cross-Talk and Innate Immune Function Presented to the University of Louisville Department of Microbiology and Immunology. 08/2008.

Complement-TLR Crosstalk and Microbial Immune Evasion. Presented to the University of Louisville Department of Microbiology and Immunology. 09/2009.

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**Mentorship:**

Amanda Brinkworth, Ph.D. Candidate (University of Louisville, Dept. of Microbiology and Immunology). 10/2005-12/2007. Role of *Chlamydial*-derived heat-shock protein in promoting tarp-induced invasion of *Chlamydia trachomatis*.

Megan McIntosh, Ph.D. Candidate (University of Louisville, Dept. of Microbiology and Immunology). 2/2009-current. Counteracting microbial immune evasion for the treatment of periodontitis.

**Teaching Experience:**

Undergraduate Teaching Assistant 1/2003-6/2003

- Principles of Systematics

Graduate Teaching Assistant. 8/2003-6/2004

- Clinical Microbiology

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**Awards:**

The Integrated Programs in Biomedical Sciences Fellowship. 08/04.

Scientific Poster Competition for Research Louisville, **First Prize**. 10/10.