

Microbial Hijacking of Complement–Toll-Like Receptor Crosstalk

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Crosstalk between complement and Toll-like receptors (TLRs) coordinates innate immunity. We report a previously unknown immune subversion mechanism involving microbial exploitation of communication between complement and TLRs. *Porphyromonas gingivalis*, a major oral and systemic pathogen with complement C5 convertase-like activity, synergizes with C5a (fragment of complement protein C5) to increase cyclic adenosine monophosphate (cAMP) concentrations, resulting in suppression of macrophage immune function and enhanced pathogen survival in vitro and in vivo. This synergy required TLR2 signaling, a pertussis toxin- and thapsigargin-sensitive C5a receptor pathway, with protein kinase A and glycogen synthase kinase-3 β as downstream effectors. Antagonistic blockade of the C5a receptor abrogated this evasive strategy and may thus have important therapeutic implications for periodontitis and atherosclerosis, diseases in which *P. gingivalis* is implicated. This first demonstration of complement-TLR crosstalk for immunosuppressive cAMP signaling indicates that pathogens may not simply undermine complement or TLRs (or both) as separate entities, but may also exploit their crosstalk pathways.

INTRODUCTION

Although traditionally perceived as an antimicrobial enzyme system in serum, complement is now recognized as a central component of host defense that affects both innate and adaptive immunity (1). The triggering of complement involves sequential activation and proteolytic cleavage of a series of serum proteins and proceeds through three distinct mechanisms, namely, the classical, lectin, or alternative pathway (1). All three pathways converge at the third component of complement (C3), which is activated by pathway-specific C3 convertases and leads to the generation of effector molecules that mediate recruitment and activation of inflammatory cells (mediated by the anaphylatoxins C3a and C5a), microbial opsonization and phagocytosis (through the action of opsonins such as C3b and iC3b), and direct lysis of targeted pathogens (through the C5b-9 membrane attack complex) (1). Crosstalk has been suggested to occur between complement and another major innate defense system, the Toll-like receptors (TLRs), to apparently coordinate the host response to infection (2–4). The TLRs constitute a family of pattern-recognition receptors that detect distinct types of structurally conserved microbial molecules, thus endowing the innate immune system with a degree of specificity. For example, TLR2 recognizes microbial lipoproteins, TLR4 detects bacterial lipopolysaccharide, and TLR5 recognizes bacterial flagellin (5). After pathogen recognition, TLR signaling is initiated by recruitment of shared or pathway-specific adaptor molecules that help propagate the signals to downstream kinases and transcription factors, such as nuclear factor κ B (NF- κ B) or interferon reg-

ulatory factor-3 [or both, depending on the activated TLR pathway(s)], leading to induction of proinflammatory and immunoregulatory responses aiming to contain the infection and instruct the development of adaptive immunity (6, 7). Not surprisingly, given their importance in fighting pathogens, complement and TLRs constitute key targets of immune evasion by microbes that cause persistent infections (8, 9). Here, we describe a previously unknown strategy of immune subversion, involving microbial exploitation of the fifth complement component (C5) for corrupting TLR-conferred immunity through complement-TLR crosstalk.

The pathogen involved in these subversive interactions, *Porphyromonas gingivalis*, is a Gram-negative anaerobic bacterium. This organism is associated with periodontitis, a prevalent oral chronic inflammatory disease, and is moreover implicated in systemic conditions such as atherosclerosis and aspiration pneumonia (10). *P. gingivalis* inhibits the complement cascade regardless of the initiation pathway involved, but, curiously, selectively generates biologically active C5a (11, 12). C5a generation by *P. gingivalis* is mediated by its Arg-specific cysteine proteinases (RgpA and RgpB gingipains), which act in a C5 convertase-like manner (11, 12). Moreover, after release of C5a from C5, the C5b remnant is proteolytically destroyed by *P. gingivalis* (11) to apparently prevent activation of the terminal complement pathway, which leads to the formation of the membrane attack complex (1). Because C5a is a powerful chemoattractant and activator of phagocytes (13), 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 (12).

Here, we show that *P. gingivalis* paradoxically uses the proinflammatory C5a to suppress immune function of macrophages through a previously unknown crosstalk mechanism between the C5a receptor (C5aR) and TLR2, the predominant TLR utilized by this organism in vitro and

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in vivo (14, 15). As far as we know, this is the first report of a pathogen capable of proactively instigating and exploiting crosstalk signaling between complement and TLRs, rather than undermining either system independently as previously shown for other microbes (8, 16).

RESULTS

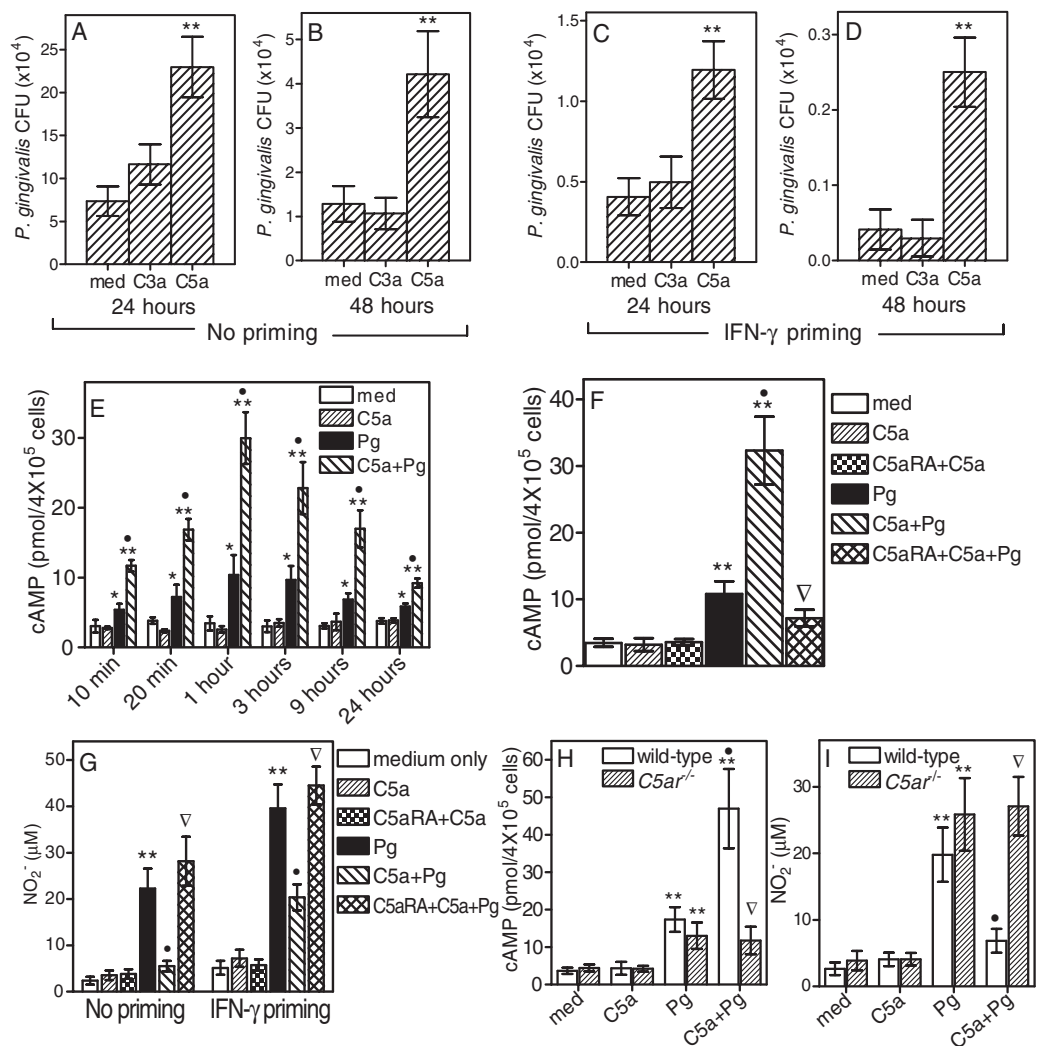
***P. gingivalis* uses C5a to subvert macrophage function**

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 (11, 12). We first examined whether C5a influences the macrophage intracellular killing of *P. gingivalis*. Strikingly, C5a, but not the related anaphylatoxin C3a, significantly promoted the ability of this pathogen to survive intracellularly in mouse macrophages (Fig. 1, A and B). This unexpected promicrobial effect of C5a was enhanced with increasing concentrations of C5a (fig. S1A) and was also observed in interferon- γ (IFN- γ)–primed macrophages (Fig. 1, C and D). The

elevated viable cell counts of *P. gingivalis* in C5a-treated macrophages could not be attributed to differences in the initial bacterial loads, because *P. gingivalis* phagocytosis was not significantly affected by the absence or presence of C5a or C3a (fig. S2A). Furthermore, the abundance of macrophage receptors that coordinately mediate *P. gingivalis* uptake, such as CD14, TLR2, and CD11b/CD18, was unaffected by C5a (fig. S2, B and C).

We next investigated the mechanism underlying C5a-mediated inhibition of the macrophage intracellular killing capacity. We hypothesized that the combined action of C5a and *P. gingivalis* on macrophages may induce immunosuppressive signaling. We first used real-time quantitative polymerase chain reaction (PCR) to determine whether C5a increases the abundance of messenger RNAs (mRNAs) encoding inhibitors of TLR signaling in *P. gingivalis*–stimulated macrophages. Although exposure to the bacteria alone increased the mRNA abundance for some of the investigated regulators, including the suppressor of cytokine signaling-1 (SOCS1), the interleukin-1 (IL-1) receptor–associated kinase M (IRAK-M), and the ubiquitin-editing enzyme A20, no synergistic or additive effects were seen in the concomitant presence of *P. gingivalis* and C5a (fig. S3).

Fig. 1. Immunosubversive effects of C5a on macrophages. (A to D) Peritoneal mouse macrophages were left untreated (A and B) or primed with IFN- γ (100 ng/ml) (C and D) overnight, washed, and incubated with *P. gingivalis* (Pg; MOI = 10:1) in the presence or absence of C3a (200 nM) or C5a (50 nM). Viable counts of internalized bacteria at 24 hours (A and C) or 48 hours (B and D) after-infection were determined by CFU enumeration. (E) Macrophages were incubated with medium only or with *P. gingivalis* in the presence or absence of C5a for the indicated times and assayed for induction of intracellular cAMP production. (F) Similar experiment as in (E), involving 1 hour of incubation and the use of a specific C5a receptor antagonist (C5aRA; 1 μ M), as indicated. (G) Unprimed or IFN- γ –primed macrophages were assayed for NO $_2^-$ after a 24-hour incubation with or without *P. gingivalis*, C5a, or C5aRA. (H to I) Similar experiments for induction of cAMP (H) and NO $_2^-$ (I) production with macrophages from wild-type or C5aR-deficient (*C5ar*^{-/-}) mice. Data are means \pm SD (n = 3 sets of macrophages) from typical experiments performed three (A to D, F, and G) or two (E, H, and I) times yielding consistent results. * P < 0.05 and ** P < 0.01 compared to medium (med) control 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.



Therefore, these regulatory molecules are not likely involved in C5a-mediated suppression of macrophage killing of *P. gingivalis*. Moreover, although induction of cyclic adenosine monophosphate (cAMP) production can induce immunosuppressive signaling (17), C5a by itself did not induce cAMP production in macrophages (Fig. 1E). Strikingly, however, C5a synergized with *P. gingivalis*, resulting in a more than threefold increase of intracellular cAMP concentrations relative to *P. gingivalis* stimulation alone (Fig. 1E). The synergy was observed as early as 10 min after cell stimulation and peaked at 1 hour, but significantly increased cAMP concentrations were sustained for at least 24 hours (Fig. 1E). This up-regulatory effect of C5a was dose-dependent (fig. S1B) and was

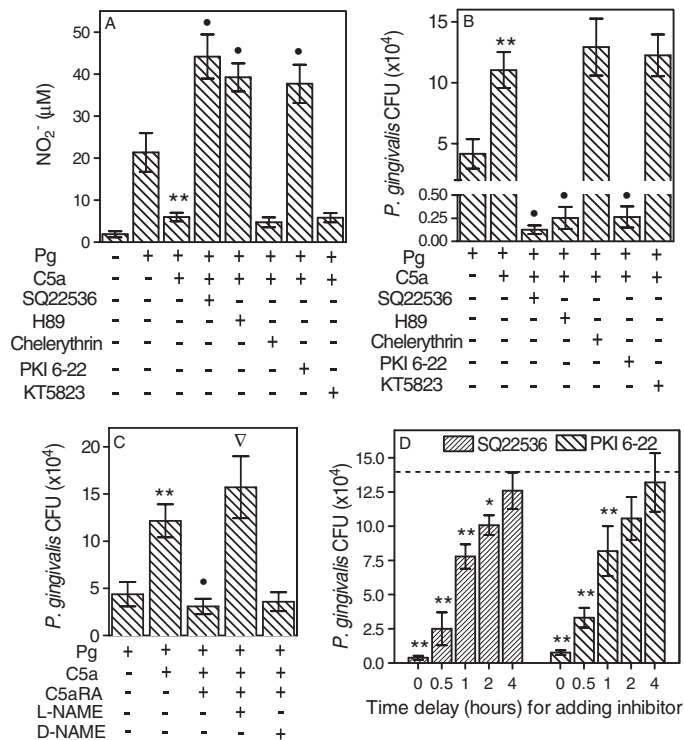


Fig. 2. C5a-mediated inhibition of nitric oxide production and promotion of *P. gingivalis* survival is cAMP and PKA dependent. (A and B) Mouse macrophages were pretreated or not with SQ22536 (cAMP synthesis inhibitor; 200 µM), H89 (PKA inhibitor; 5 µM), chelerythrin (protein kinase C inhibitor; 5 µM), PKI 6-22 (peptide inhibitor of PKA; 1 µM), or KT5823 (peptide inhibitor of protein kinase G; 1 µM), and then infected with *P. gingivalis* (MOI = 10:1) with or without C5a (50 nM), as indicated. (C) Macrophages were pretreated with 1 mM L-NAME (or D-NAME), 1 µM 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. NO₂⁻ production (A) and viable counts of internalized bacteria (B to D) were determined at 24 hours after infection. In (D), the dashed line indicates *P. gingivalis* CFUs in the absence of inhibitors [13.7 ± 2.7 (× 10⁴) CFUs]. Results are means ± SD (n = 3 sets of macrophages) 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.

abrogated by a C5aR antagonist (C5aRA), the cyclic hexapeptide AcF(OP(D)ChaWR) [acetylated phenylalanine-(ornithine-proline-(D) cyclohexylalanine-tryptophan-arginine)] (Fig. 1F), indicating that C5a acted through the classic C5aR (CD88), rather than the alternative C5a-like receptor 2.

Given that *P. gingivalis* is resistant to killing by the oxidative burst (18), we investigated whether C5a interferes with induction of nitric oxide production as a possible mechanism for its promicrobial effect. The underlying rationale was that *P. gingivalis* is sensitive to nitric oxide-mediated killing (19, 20). Indeed, C5a significantly inhibited through a C5aR-dependent mechanism the production of nitric oxide in *P. gingivalis*-stimulated macrophages, even in cells primed with IFN-γ (Fig. 1G). The requirement for C5aR in C5a-mediated augmentation of cAMP production and suppression of nitric oxide production in *P. gingivalis*-challenged macrophages was confirmed by lack of these effects in C5aR-deficient (*C5ar^{-/-}*) macrophages (Fig. 1, H and I, respectively). The inhibitory effect of C5a on nitric oxide production was dose-dependent (fig. S4, A and B), although it progressively declined with increasing delay in adding C5a to *P. gingivalis*-infected macrophages (fig. S4, C and D), suggesting a requirement early in the infection process for crosstalk between C5a- and *P. gingivalis*-induced signaling. On the other hand, when C5a was added together with *P. gingivalis*, the inhibitory effect of C5a was maintained for at least 48 hours (fig. S4, E and F). Together, these findings suggest that C5aR activation by C5a results in suppression of *P. gingivalis* intracellular killing, which correlates with elevating cAMP concentrations and reducing nitric oxide production.

C5a immunosubversive effects are strictly dependent on cAMP-PKA signaling

We investigated whether the C5a-mediated inhibition of nitric oxide production depends on the ability of C5a to stimulate synergistic elevation of cAMP concentrations. Indeed, the inhibitory effect of C5a on nitric oxide production was reversed in macrophages pretreated with inhibitors of cAMP synthesis (SQ22536) or of protein kinase A (PKA) (H89 and PKI 6-22), but not of other kinases, such as protein kinase C (by chelerythrin) or G (by KT5823) (Fig. 2A), indicating that the C5a effect is mediated by cAMP-dependent PKA signaling. Furthermore, the increase in nitric oxide concentrations by inhibitors of cAMP synthesis or of PKA was linked to significantly reduced intracellular survival of *P. gingivalis* in those same cells (Fig. 2B). Moreover, macrophage pretreatment with C5aRA counteracted the protective effect of C5a on *P. gingivalis* intracellular viability, whereas L-NAME (*N^G*-nitro-L-arginine methyl ester, an inhibitor of nitric oxide synthesis), but not an inactive enantiomer control (D-NAME, *N^G*-nitro-D-arginine methyl ester), mimicked C5a and overrode the C5aRA effect (Fig. 2C). The ability of inhibitors of cAMP synthesis or of PKA to reverse the immunosuppressive C5a effect progressively declined with increasing delay in their addition to the culture system (Fig. 2D). 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 to disable *P. gingivalis*-challenged macrophages (fig. S4, C and D).

C5aR signaling is exploited in vivo to inhibit nitric oxide production and promote microbial survival

To determine if C5aR signaling also promotes *P. gingivalis* virulence 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 after infection, the peritoneal lavage fluid from C5aRA-treated mice contained significantly lower *P. gingivalis* colony-forming units

(CFUs) compared to untreated mice (>95% reduction) (Fig. 3A). Consistent with this, *C5ar^{-/-}* mice controlled *P. gingivalis* infection better than did wild-type mice (Fig. 3A). In addition, wild-type mice were bacteremic for *P. gingivalis* (four out of five mice in this group had positive blood cultures 24 hours after infection), whereas no bacteremia could be detected in *C5ar^{-/-}* mice (table S1), 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 detection of *P. gingivalis* CFUs in homogenates of several organs examined (spleen, kidney, liver, and lungs) from either *C5ar^{-/-}* or wild-type mice (table S1). The enhanced clearance of *P. gingivalis* in C5aRA-treated mice correlated with increased nitric oxide production (relative to untreated mice), and both effects were counteracted by L-NAME treatment (Fig. 3, 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.

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 Ca^{2+} may potentiate induction of cAMP production by the appropriate stimuli (21). If the synergistic effect of C5a on induction of cAMP production (Fig. 1E) depends on its Ca^{2+} -mobilizing activity, then this synergy should be inhibited by thapsigargin, which inhibits the endoplasmic reticulum Ca^{2+} -adenosine triphosphatase (ATPase) and blocks the C5a-induced intracellular Ca^{2+} response (22). Indeed, macrophage pretreatment with thapsigargin abrogated the synergistic C5a effect on *P. gingivalis*-induced cAMP production, whereas EGTA, which chelates extracellular Ca^{2+} , had a relatively minimal and statistically insignificant effect (Fig. 4A). Significant reversal of the C5a effect on induction of cAMP production was also seen in cells pretreated with pertussis toxin (Fig. 4A), suggesting a requirement for G_{α_i} -coupled C5aR signaling (23).

In the absence of C5a, the ability of *P. gingivalis* to induce cAMP production depends on its interaction with the CXC-chemokine receptor 4 (CXCR4) (20). Thus, we initially speculated that the synergistic C5a effect on the induction of cAMP production could involve crosstalk between C5aR and CXCR4. Although CXCR4 blockade by AMD3100 [at 1 μ g/ml, which completely inhibits the CXCR4-*P. gingivalis* interaction (20)] modestly attenuated the synergistic C5a effect on cAMP production, there was still a more than sixfold difference in cAMP concentrations between C5a-treated and untreated macrophages (Fig.

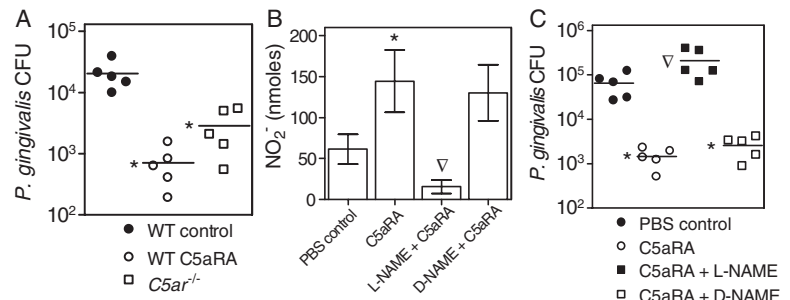
4B). Moreover, *P. gingivalis* did not increase intracellular cAMP concentrations in CXCR4-transfected Chinese hamster ovary (CHO)-K1 cells, although it induced cAMP production in cells cotransfected with CXCR4 and TLR2 (fig. S5). Therefore, CXCR4 is not directly involved in induction of cAMP production but cooperates in that regard with TLR2, which on its own weakly induces cAMP production (fig. S5).

Indeed, the ability of C5a to synergistically induce cAMP production and activate PKA was seen in *P. gingivalis*-stimulated macrophages from wild-type, but not *Tlr2^{-/-}* mice (Fig. 4, C and D). However, the inherent capacity of *Tlr2^{-/-}* macrophages to increase intracellular cAMP concentrations and activate PKA was confirmed by directly activating adenylate cyclase with forskolin (Fig. 4, C and D). This concept of C5aR-TLR2 crosstalk for synergistic cAMP-dependent PKA activation is consistent with additional findings from an in vivo experiment. Indeed, PKA activity was significantly lower in freshly explanted peritoneal macrophages from *P. gingivalis*-infected mice with TLR2 or C5aR deficiency, but not those from mice with TLR4 or C3aR deficiency or from wild-type mice (Fig. 4E).

We also showed that another synergistic interaction downstream of this receptor crosstalk involved PKA-dependent phosphorylation of glycogen synthase kinase-3 β (GSK3 β) on Ser⁹ (Fig. 4F), an event that inactivates this kinase that would otherwise promote cell activation (24). Indeed, although C5a or *P. gingivalis* by themselves only slightly increased phosphorylation of Ser⁹ of GSK3 β , their combination displayed a synergistic effect that was inhibited by PKI 6-22 (but not by PD98059, an inhibitor of mitogen-activated protein kinase kinase) (Fig. 4F). The GSK3 β inhibitor SB216763 mimicked the inhibitory effect of C5a on *P. gingivalis*-induced inducible nitric oxide synthase (iNOS) abundance and nitric oxide production, as did the PKA agonist 8-Br-cAMP (Fig. 4G). Thus, GSK3 β appears to regulate iNOS and nitric oxide production downstream of PKA in C5a-treated and *P. gingivalis*-challenged macrophages.

Crosstalk between C5aR and TLR2 is also consistent with confocal microscopy findings revealing colocalization of the two receptors in *P. gingivalis*-stimulated macrophages (Fig. 4H), and with fluorescence resonance energy transfer (FRET) experiments indicating that C5aR, TLR2, and *P. gingivalis* come into molecular proximity (Fig. 4I). Indeed, FRET analysis revealed significant energy transfer between Cy3-labeled C5aR and Cy5-labeled TLR2 in *P. gingivalis*-stimulated but not resting macrophages (Fig. 4I). No significant energy transfer was detected between Cy3-labeled C5aR and Cy5-labeled TLR5 or major histocompatibility complex (MHC) class I (controls) under the same conditions (Fig. 4I). Moreover, significant energy transfer was observed between fluorescein isothiocyanate (FITC)-labeled *P. gingivalis* and tetramethyl rhodamine isothiocyanate (TRITC)-labeled C5aR or TLR2 (but not TLR5 or

Fig. 3. *P. gingivalis* exploits C5aR signaling to inhibit nitric oxide production and promote its survival in vivo. (A) Wild-type (WT) mice were intraperitoneally pretreated 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^{-/-}*), with 5×10^7 CFU *P. gingivalis*. (B and C) Wild-type mice were intraperitoneally pretreated 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*. Peritoneal fluid was collected 24 hours after infection and used to determine viable *P. gingivalis* CFU (A and C) and NO_2^- 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.



MHC class I) (Fig. 4I). However, unlike TLR2, which can directly be engaged by *P. gingivalis* (14, 25), C5aR appeared to associate indirectly with *P. gingivalis* in a TLR2-dependent way; indeed, the *P. gingivalis*-C5aR FRET association was abrogated in *Tlr2*^{-/-} macrophages (Fig. 4I).

FRET analysis further revealed that in *P. gingivalis*-challenged macrophages, C5aR also associates with CXCR4 (Fig. 4I), suggesting co-association of all three receptors (CXCR4, TLR2, and C5aR). These

interactions likely occur in lipid rafts because all three receptors (but not TLR5 or MHC class I) come within FRET proximity to an established lipid raft marker (*G*_{M1} ganglioside) in *P. gingivalis*-stimulated macrophages, unless rafts are disrupted by methyl- β -cyclodextrin treatment (fig. S6). Although the C5aR-TLR2 crosstalk can proceed independently of CXCR4 and potentially increase cAMP production (Fig. 4B), maximal cAMP induction requires the cooperation of all three receptors (Fig. 4J).

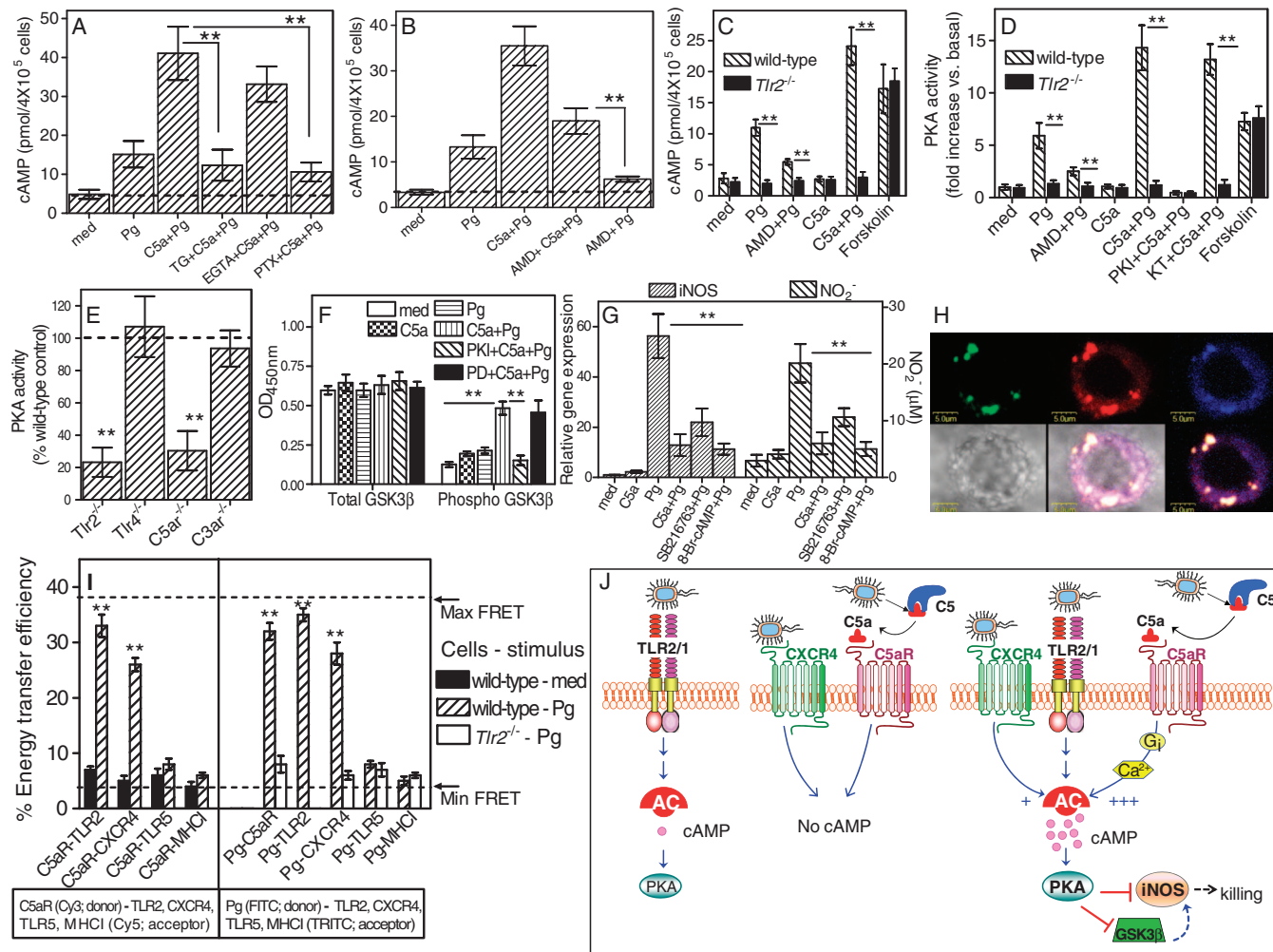


Fig. 4. Synergistic activation of the cAMP-PKA pathway requires C5aR-TLR2 crosstalk. (A to D) Macrophages pretreated with 1 μ M thapsigargin (TG), 5 mM EGTA, pertussis toxin (100 ng/ml) (PTX) (A) or AMD3100 (1 μ g/ml) (B to D) were stimulated with *P. gingivalis* (MOI = 10:1; 1 hour) with or without 50 nM C5a and assayed for cAMP concentrations (A to C) or PKA activity (D). PKA assay specificity was confirmed with PKI-6-22 and an irrelevant kinase inhibitor (KT5823). Forskolin (20 μ M; 10 min) served as positive control in experiments with *Tlr2*^{-/-} macrophages (C and D). (E) PKA activities in freshly explanted peritoneal macrophages from *P. gingivalis*-infected mice (activities of indicated receptor-deficient cells are shown as percent wild-type activity). (F) Macrophages pretreated with 1 μ M PKI-6-22 or 25 μ M PD98059 (PD; control) were stimulated with *P. gingivalis*, with or without C5a, and assayed for GSK3 β Ser⁹-phosphorylation and total GSK3 β . (G) Macrophages stimulated with *P. gingivalis* with or without C5a (50 nM), SB216763 (10 μ M),

or 8-Br-cAMP (100 μ M) were assayed for iNOS (4 hours) or NO₂⁻ (24 hours). (H) Confocal colocalization of *P. gingivalis* (green), C5aR (red), and TLR2 (blue). Bottom right, merged image. (I) FRET between the indicated donors and acceptors measured from the increase in donor (Cy3 or FITC) fluorescence after acceptor (Cy5 or TRITC) photobleaching. Data are means \pm SD [*n* = 3 sets of macrophages except for (E), *n* = 5 mice] from typical experiments performed at least twice with consistent results. **P* < 0.05; ***P* < 0.01 between the indicated groups or compared to controls (E and I). (J) *P. gingivalis* induces 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 β and impairs iNOS-dependent killing.

DISCUSSION

Crosstalk between the complement system and the TLRs seems essential to appropriately coordinate the early innate response to infection (2–4). Here, we addressed the intriguing possibility that at least some of the interplay between complement and TLR 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 (26). Therefore, we propose that this pathogen has evolved a subversive C5aR-TLR2 crosstalk mechanism for blunting the TLR2 antimicrobial response (Fig. 4J) as an alternative to direct TLR2 antagonism. Notably, *P. gingivalis* does not rely on immunological mechanisms to activate C5aR, because it can activate this receptor through gingipain-mediated local generation of C5a (11) (Fig. 4J). We confirmed and expanded the biochemical demonstration of C5a generation by purified gingipains acting on purified C5 substrate (11), by estimating that *P. gingivalis* generates high amounts of C5a (32.7 ± 4.3 nM) after a 30-min incubation in heat-inactivated human serum (fig. S7). Notably, unlike C5a, C3a is extensively degraded and inactivated by *P. gingivalis* (11). Because C3a (but not C5a) exerts direct bactericidal effects (27), C3a destruction by *P. gingivalis* may serve to protect this pathogen.

The striking ability of C5a to synergize to induce cAMP production with *P. gingivalis* in a pertussis toxin–sensitive and TLR2-dependent way could be explained as follows. The Gi β subunits, which are released upon activation of the pertussis toxin-sensitive G α i subunit, can regulate adenylate cyclase activity, either positively or negatively depending on the enzyme isoform (28). Thus, although Gi β cannot stimulate adenylate cyclase by themselves, they can increase the activity of several adenylate cyclase isoforms in the presence of an appropriate stimulus. Such stimulus is apparently provided by *P. gingivalis* activation of TLR2. Moreover, the adenylate cyclase isoforms that are positively regulated by Gi β are not those that are sensitive to the inhibitory action of G α i (28). Because the ability of C5a to synergize with *P. gingivalis* to induce cAMP production also depends on intracellular Ca²⁺, Gi β may mediate their stimulatory effects on AC activity through their Ca²⁺-mobilizing effects.

A major mechanism underlying the regulatory effects of cAMP on cell activation involves the ability of PKA to phosphorylate the cAMP response element-binding protein (CREB), which effectively competes with the p65 subunit of NF- κ B for limiting amounts of common transcriptional cofactors (17). Besides being under the control of NF- κ B, iNOS is additionally regulated by IFN- γ ; however, PKA also inhibits IFN regulatory factor-1 (IRF-1), which is required for the synergistic IFN- γ contribution to iNOS transcription (29, 30). Moreover, PKA can phosphorylate and inactivate GSK3 β (Fig. 4F), thus abrogating its stimulatory effect on proinflammatory gene expression (24). Because PKA activation causes greater iNOS inhibition than GSK3 β inactivation (Fig. 4G), it is likely that PKA may also inhibit iNOS in a GSK3 β -independent way (Fig. 4J).

Although modest TLR-induced cAMP production may control excessive proinflammatory signaling, sustained high concentrations 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, because C5a actually enhanced *P. gingivalis*-induced IL-6 production (fig. S8). This enhancement of IL-6 production is not paradoxical, even though cAMP-

dependent PKA signaling inhibits NF- κ B–dependent transcription (31). This is because the *IL-6* gene contains cAMP-responsive elements and is not regulated solely by NF- κ B; indeed, cAMP and agents that trigger cAMP synthesis can induce IL-6 production even if the NF- κ B sites in the *IL-6* promoter are mutated (32, 33). Consistent with these observations, cholera toxin (which inhibits NF- κ B but increases cAMP concentrations) synergistically enhances lipopolysaccharide-induced IL-6 production, although it inhibits lipopolysaccharide induction of other proinflammatory cytokines [such as tumor necrosis factor- α (TNF- α), IL-12, and macrophage inflammatory protein-1 α] (34). Similarly, forskolin and other cAMP-inducing agents increase IL-6 production, although they inhibit TNF- α production (35, 36). Our findings, therefore, that C5a and *P. gingivalis* synergize for production of both cAMP and IL-6, while at the same time inhibiting induction of iNOS production that is dependent on NF- κ B (37), are consistent with the above reports. This sophisticated subversive crosstalk instigated by *P. gingivalis* (Fig. 4J) serves in lieu of “built-in” adenylate cyclase which is not present in this bacterium, in contrast to *Bordetella pertussis* which disables human or mouse phagocytes by means of its own adenylate cyclase (38).

Macrophages can interact with *P. gingivalis* not only in periodontal tissues, but also in the setting of systemic inflammatory diseases such as atherosclerosis (10, 18, 39). Our previous findings that *P. gingivalis* persists intracellularly in macrophages for at least 72 hours (40) were confirmed by an independent group, which additionally showed that up to 25% of the cells undergo necrosis by 72 hours and release cellular contents (41). It is thus conceivable that viable *P. gingivalis* could be released from necrotic macrophages, especially in the presence of C5a, which in turn promotes its intracellular persistence. This possibility becomes intriguing in view of epidemiological and mechanistic links between periodontitis and atherosclerosis (10, 39). However, whether the documented localization of viable *P. gingivalis* bacteria in atherosclerotic plaques (42) 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 (43, 44).

C5aR activation in macrophages also inhibited TLR4-induced expression of mRNAs encoding IL-12p35, IL-12/IL-23p40, and IL-23p19, and production of IL-12p70 and IL-23 protein, through C5a-induced phosphatidylinositol 3-kinase (PI3K) and extracellular signal-regulated kinase 1/2 (ERK1/2) signaling (2, 3, 45). The physiological significance of these C5a regulatory effects is likely to attenuate potential tissue damage mediated by various T cell effector subsets (such as Th1 and Th17, which are regulated by IL-12 and IL-23, respectively), as seen in various pathological inflammatory conditions (46). 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 (4).

In summary, this work constitutes the first report of complement-TLR crosstalk for synergistic induction of cAMP production that 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 such as atherosclerosis. Because C5a can be generated by both complement and noncomplement C5 convertases that also include microbial enzymes (11, 47, 48), it becomes important to identify other

pathogens that exploit C5a-mediated subversive crosstalk signaling with TLRs. This will have important implications for the development of new counterstrategies 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 exemplified by *P. gingivalis*, to also exploit their communication hubs.

MATERIALS AND METHODS

Reagents

SQ22536, H89, SB216367, 8-Br-cAMP, AMD3100, forskolin, L-NAME, D-NAME, 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 IFN- γ was from the R&D Systems. Mouse C5a was purchased from Cell Sciences or R&D Systems and C3a from R&D Systems. The cyclic hexapeptide AcF(OP(D)ChaWR), a specific and potent C5a receptor (CD88) antagonist, was synthesized in the laboratory of one of the coauthors (J.D.L.), as previously described (49, 50). C5a and C3a were used at concentrations up to 100 and 200 nM, respectively, which are widely used in *in vitro* experiments (2, 3). Moreover, these concentrations are consistent with observations that under inflammatory conditions, C5a and C3a may reach serum concentrations as high as 100 and 400 nM, respectively, although even higher concentrations may be generated at local sites of inflammation (13, 51). All reagents were used at optimal concentrations determined in preliminary or published studies by our laboratories (20, 50, 52). When appropriate, dimethyl sulfoxide (DMSO) was included in medium controls and its final concentration was $\leq 0.2\%$.

Bacteria and mammalian cells

P. gingivalis ATCC (American Type Culture Collection) 33277 was grown anaerobically from frozen stocks on modified Gifu anaerobic medium (GAM)-based blood agar plates for 5 to 6 days at 37°C, followed by anaerobic subculturing for 18 to 24 hours at 37°C in modified GAM broth (Nissui Pharmaceutical). Thioglycollate-elicited macrophages were isolated from the peritoneal cavity of wild-type or mice deficient in TLR2, TLR4, C3aR, or C5aR (The Jackson Laboratory) (3, 14), in compliance with established federal guidelines and institutional policies. The macrophages were cultured at 37°C and 5% CO₂ in RPMI 1640 (Invitrogen) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, penicillin G (100 U/ml), streptomycin (100 μ g/ml), and 0.05 mM 2- β -mercaptoethanol. None of the experimental treatments, including treatments with C5a up to 100 nM, affected cell viability (as monitored by the CellTiter-Blue assay; Promega) compared to medium-only treatments.

Intracellular survival assay

The viability of phagocytosed *P. gingivalis* was monitored by an antibiotic protection-based intracellular survival assay, as previously described (40). Briefly, mouse peritoneal macrophages were allowed to phagocytose *P. gingivalis* [multiplicity of infection (MOI) = 10:1; 5×10^6 bacteria and 5×10^5 cells] for 30 min at 37°C. This was followed by washing to remove extracellular nonadherent bacteria and a 1-hour treatment with antibiotics [gentamicin (300 μ g/ml) and metronidazole (200 μ g/ml)] to eliminate residual or extracellular adherent bacteria. The macrophages were subsequently cultured overnight (for a total of 24 hours) or for 48

hours, then immediately washed and lysed in sterile distilled water. Viable counts of internalized *P. gingivalis* were determined by plating serial dilutions of macrophage lysates on blood agar plates subjected to anaerobic culture (40).

Cell signaling and activation assays

Induction of nitric oxide production was assessed by measuring the amount of NO₂⁻ (stable metabolite of nitric oxide) in stimulated culture supernatants with a Griess reaction-based assay kit (R&D Systems), as previously performed (20). cAMP concentrations in activated cell extracts were measured with a cAMP enzyme immunoassay kit (Cayman Chemical) (52). PKA activity in lysates of activated cells was determined with the ProFluor PKA assay, according to the manufacturer's instructions (Promega) (20). Phosphorylation of GSK3 β on Ser⁹ and total GSK3 β were monitored with FACE GSK3 β ELISA kits (Active Motif).

In vivo infection

Upon intraperitoneal infection of mice with *P. gingivalis* (5×10^7 CFU), peritoneal lavage was performed 24 hours after infection and the peritoneal fluid was used to enumerate recovered CFU (after anaerobic growth on blood agar plates) and measure production of NO₂⁻ (20). All animal procedures were approved by the Institutional Animal Care and Use Committee and performed in compliance with established federal and state policies.

Quantitative real-time PCR

Gene expression in resting or activated mouse macrophages was quantified with quantitative real-time PCR. Briefly, RNA was extracted from cell lysates with the PerfectPure RNA cell kit (5 Prime, Fisher) and quantified by spectrometry at 260 and 280 nm. The RNA was reverse-transcribed with the High-Capacity cDNA Archive kit (Applied Biosystems) and quantitative real-time PCR with complementary DNA (cDNA) was performed with the ABI 7500 Fast System according to the manufacturer's protocol (Applied Biosystems). TaqMan probes, sense primers, and antisense primers for expression of a housekeeping gene (GAPDH) or iNOS (or genes shown in fig. S3) were purchased from Applied Biosystems.

Confocal microscopy

To examine colocalization 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 antibody against C5aR plus allophycocyanin-labeled antibody to TLR2, and mounted with coverslips for imaging on an Olympus FV500 confocal microscope (40).

Fluorescence resonance energy transfer

After 10 min of stimulation with *P. gingivalis* at 37°C, mouse macrophages were labeled with a mixture of Cy3-conjugated (donor) and Cy5-conjugated (acceptor) antibodies, as indicated in Fig. 4I. In other experiments shown in Fig. 4I, 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 (14, 53). 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; their values are denoted by

dashed lines in Fig. 4I. The conjugation of antibodies to Cy3 or Cy5 was performed with kits from Amersham Biosciences.

Statistical analysis

Data were evaluated by analysis of variance and the Dunnett multiple-comparison test with the InStat program (GraphPad Software). Where appropriate (comparison of two groups only), two-tailed *t* tests were performed. *P* < 0.05 was considered to be significant. All experiments were performed at least twice for verification.

SUPPLEMENTARY MATERIALS

www.sciencesignaling.org/cgi/content/full/3/109/ra11/DC1

Table S1. Detection of *P. gingivalis* in blood and internal organs of wild-type and C5aR-deficient (*C5ar^{-/-}*) mice after intraperitoneal infection.

Fig. S1. C5a dose-dependently promotes the intracellular survival of *P. gingivalis* and the cAMP response.

Fig. S2. C5a does not affect *P. gingivalis* phagocytosis.

Fig. S3. Relative mRNA abundance of negative regulators of TLR signaling in *P. gingivalis*-stimulated macrophages in the absence or presence of C5a.

Fig. S4. C5a inhibits nitric oxide production in a dose- and time-dependent way

Fig. S5. TLR2-dependent cAMP production by *P. gingivalis*.

Fig. S6. Association of TLR2, C5aR, and CXCR4 with G_{M1} (lipid raft marker) in *P. gingivalis*-stimulated macrophages.

Fig. S7. Generation of C5a by *P. gingivalis* from heat-inactivated human serum.

Fig. S8. Up-regulation of IL-6 production by C5a in *P. gingivalis*-stimulated macrophages.

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