Received Date : 14-Mar-2016

Revised Date : 08-Apr-2016

Accepted Date : 11-Apr-2016

Article type : Original Article

Differential capacity for complement receptor-mediated immune evasion by *Porphyromonas gingivalis* depending on the type of innate leukocyte

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Running Title: *P. gingivalis*, complement and dendritic cells

Keywords: Immune evasion; intracellular killing; *P. gingivalis;* dendritic cells; complement; C3aR; C5aR1; C5aR2; CR3.

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SUMMARY

The complement system plays a central role in immunity and inflammation, although certain pathogens can exploit complement to undermine protective immunity. In this context, the periodontal keystone pathogen *Porphyromonas gingivalis* (Pg) was previously shown by our group to evade killing by neutrophils or macrophages through exploitation of complement C5a receptor 1 (C5aR1) and complement receptor-3 (CR3). Here, we examined whether Pg uses complement receptors to also subvert killing by dendritic cells. In line with earlier independent studies, intracellular viable Pg bacteria could be recovered from mouse bone marrow-derived dendritic cells (BMDC) or human monocyte-derived dendritic cells (MDDC) exposed to the pathogen. However, in the presence of C5a, the intracellular survival of Pg was significantly decreased in a C5aR1-dependent way. Further work using wild-type and receptor-knockout BMDC showed that, in the presence of C3a, the C3a receptor (C3aR) similarly enhanced the intracellular killing of Pg. In contrast, C5aR2, an alternative receptor for C5a (G protein-coupled receptor 77), was associated with increased intracellular Pg viable counts, consistent with the notion that C5aR2 functions as a negative regulator of C5aR1 activity. Moreover, Pg failed to utilize CR3 as a phagocytic receptor in BMDCs, in contrast to our earlier findings in macrophages where CR3-mediated uptake promotes Pg survival. Collectively, these data show that complement receptors mediate cell-type-specific effects

on how innate leukocytes handle Pg, which appears to exploit complement to preferentially evade those cells (neutrophils and macrophages) that are most often encountered in its predominant niche, the periodontal pocket.

INTRODUCTION

Porphyromonas gingivalis is a gram-negative anaerobic bacterium that is strongly associated with human periodontitis (Hajishengallis & Lamont, 2012; Hong *et al.*, 2015). Extensive research over the past three decades have identified a number of documented or putative virulence factors of *P. gingivalis* that are thought to contribute to its persistence in the periodontal pocket (Bostanci & Belibasakis, 2012; Darveau *et al.*, 2012; Hajishengallis & Lamont, 2014; Yilmaz, 2008). More recent work has started to elucidate how this bacterium integrates its virulence traits to enhance the pathogenic potential, or nososymbiocity, of polymicrobial communities (Hajishengallis & Lamont, 2016; Lamont & Hajishengallis, 2015). In this regard, studies in mice have shown that the capacity of *P. gingivalis* to induce periodontitis requires the indigenous microbiota, which is rendered dysbiotic in the presence of *P. gingivalis* (Hajishengallis *et al.*, 2011; Maekawa *et al.*, 2014). The aptitude of *P. gingivalis* to orchestrate inflammatory disease through community-wide effects, while being a low-abundance constituent of periodontitis-associated communities in humans and animal models (Abusleme *et al.*, 2013; Hajishengallis *et al.*, 2011), has prompted its designation as a keystone pathogen (Darveau, 2009; Darveau *et al.*, 2012; Hajishengallis *et al.*, 2012).

Although centrally involved in immunity and inflammation, the complement system can be subverted by various pathogens to promote their adaptive fitness in the mammalian host (Hajishengallis & Lambris, 2011; Lambris *et al.*, 2008). The triggering of the complement cascade proceeds via distinct mechanisms (classical, lectin, or alternative), all of which converge at the third complement component (C3) (Ricklin *et al.*, 2010). C3 activation by pathway-specific C3 convertases leads to the generation of effector molecules involved in (a) the recruitment and activation of inflammatory cells (e.g., the C3a and C5a anaphylatoxins that activate specific G-protein–coupled

receptors, C3a receptor [C3aR] and C5a receptor 1 [C5aR1; CD88], respectively); (b) microbial opsonization and phagocytosis (*e.g.*, through the C3b opsonin); and (c) direct lysis of targeted susceptible bacteria (by means of the C5b-9 membrane attack complex) (Ricklin *et al.*, 2010). An alternative but quite enigmatic receptor for C5a is the C5aR2 (also known as C5a-like receptor 2 or GPR77), which has been assigned both regulatory and proinflammatory roles, depending on specific context (Gerard *et al.*, 2005; Li *et al.*, 2013; Ward, 2009).

Mechanistic studies have shown that the subversion of complement is fundamental to the ability of *P. gingivalis* to modulate innate immunity and instigate quantitative and qualitative alterations in the indigenous microbiota, which can thereby cause inflammatory bone loss in the oral cavity (Hajishengallis *et al.*, 2011; Maekawa *et al.*, 2014). In this context, we have previously shown that *P. gingivalis* can protect itself and bystander bacteria by interfering with leukocyte killing mechanisms while promoting inflammation, thereby contributing to dysbiosis (Liang *et al.*, 2011; Maekawa *et al.*, 2014; Wang *et al.*, 2010). In neutrophils, the most common leukocyte recruited to periodontal pockets (Delima & Van Dyke, 2003; Hajishengallis *et al.*, 2016), *P. gingivalis* inhibits an antimicrobial Toll-like receptor (TLR)2–MyD88 pathway through proteasomal degradation of MyD88, whereas it stimulates a proinflammatory TLR2–phosphoinositide 3-kinase (PI3K) pathway (Maekawa *et al.*, 2014). The TLR2–PI3K pathway additionally suppresses RhoA GTPase-dependent actin polymerization and blocks phagocytosis in both human and mouse neutrophils (Maekawa *et al.*, 2014). These subversive pathways strictly require an intimate crosstalk between TLR2 and C5aR1 (Maekawa *et al.*, 2014).

Though minimally present in periodontal pockets, macrophages can readily encounter periodontal bacteria that have invaded into the gingival connective tissue (Delima & Van Dyke, 2003). In this regard, the generation of nitric oxide is a key effector molecule enabling the intracellular killing of pathogens by the macrophage (Nathan, 2006). Intriguingly, *P. gingivalis* can interfere with this antimicrobial function by inhibiting the expression of the inducible nitric oxide

synthase (iNOS) through a cAMP- and protein kinase A (PKA)-dependent mechanism (Wang *et al.*, 2010). Maximal induction of the cAMP response requires functional co-association and activation of three receptors, TLR2, C5aR1, and chemokine C-X-C receptor 4, by *P. gingivalis* (Hajishengallis *et al.*, 2008; Wang *et al.*, 2010). Pharmacological blockade of C5aR1 leads to significantly diminished levels of intracellular cAMP and greatly facilitates the killing of *P. gingivalis* (Wang *et al.*, 2010).

Therefore, despite using different mechanisms in neutrophils and macrophages, *P. gingivalis* exploits C5aR1 to evade killing by these leukocytes. In macrophages, *P. gingivalis* additionally induces TLR2 inside-out signaling that transactivates the high-affinity binding state of complement receptor 3 (CD11b/CD18), thereby allowing *P. gingivalis* to bind CR3 via its FimA fimbriae for a relatively safe entry into macrophages (Hajishengallis *et al.*, 2006; Wang *et al.*, 2007). Indeed, when macrophages phagocytose *P. gingivalis* by alternative receptors (*i.e.*, when CR3 is pharmacologically blocked or genetically ablated), their intracellular killing capacity is markedly potentiated (Wang *et al.*, 2007). These findings are consistent with observations that CR3 is not linked to vigorous microbicidal mechanisms (Lowell, 2006; Vieira *et al.*, 2002).

In this paper, we determined whether *P. gingivalis* uses receptors of the complement system, including C5aR1 or CR3, to manipulate dendritic cells, specialized antigen-presenting cells that internalize and process microbes and link innate and adaptive immunity (Pulendran, 2015). In comparison to neutrophils and macrophages, dendritic cells are not as potent in pathogen clearance (Silva). Surprisingly, however, we found that dendritic cells were not subverted by Pg through complement receptors, which actually facilitated the intracellular killing of Pg.

Materials and Methods

Reagents

Human or mouse C5a and mouse C3a were purchased from R&D Systems. A C5aR1 antagonist (C5aR1A; PMX53), the cyclic hexapeptide Ac-F[OP(D)Cha-WR] (acetylated phenylalanine–[ornithyl-proline-(D)cyclohexylalanine-tryptophyl-arginine]) was synthesized as previously described (Finch *et al.*, 1999). A mAb to CD11b (clone M1/70) and its isotype controls obtained from eBioscience. Cytochalasin D was from Sigma-Aldrich. Reagents used for cell culture and differentiations are mentioned in the relevant subsection. All reagents were used at optimal concentrations determined in preliminary experiments or published studies by our group (Hajishengallis *et al.*, 2006; Liang *et al.*, 2011; Wang *et al.*, 2010).

Cell isolation and culture

Bone marrow-derived dendritic cells (BMDC) were generated as we previously described (Liang *et al.*, 2009) based on the original method developed by Lutz et al (Lutz *et al.*, 1999). Briefly, bone marrow cells harvested from femur and tibia of 8-12-week-old mice were plated at $2x10^5$ cells/ml and cultured at 37°C and 5% CO_2 atmosphere, in complete RPMI (RPMI 1640 containing 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 10 mM HEPES, 100 units/ml penicillin G, 100 µg/ml streptomycin, and 0.05 mM 2-mercaptoethanol; Life Technologies) supplemented with 20 ng/ml recombinant murine GM-CSF (Peprotech). The nonadherent cells were harvested on day 8 and purified by positive selection using anti-CD11c microbeads (Miltenyi Biotec). For killing assays, the generated BMDC were cultured in complete RMPI without the antibiotics. Thioglycolate-elicited macrophages were isolated from the peritoneal cavity of C57BL/6 mice (The Jackson Laboratory), as previously described (Hajishengallis *et al.*, 2005b). DC1.2 cells, a mouse dendritic cell line (Shen *et al.*, 1997), were kindly provided by Dr. Kenneth Rock (University of Massachusetts Medical School).

Cells were cultured in complete RPMI at 37°C and 5% CO_2 atmosphere. Human monocytes were purified from peripheral blood upon centrifugation over NycoPrep 1.068, and incidental nonmonocytes were magnetically depleted as previously described (Harokopakis *et al.*, 2006). To generate monocyte-derived macrophages (MDM), monocytes were incubated in complete RPMI medium supplemented with 10 ng/ml recombinant (r)GM-CSF (Pepro Tech) for 6 days (van der Does *et al.*, 2010). Monocyte-derived DCs (MDDC) were generated as described by Jotwani et al (Jotwani *et al.*, 2001). Briefly, monocytes were incubated in complete RPMI medium supplemented with 100 ng/ml rGM-CSF and 25 ng/ml rIL-4 (Pepro Tech, Rocky Hill, NJ) for 6–8 days. After confirming the immature DC phenotype by flow cytometry ($CD14^{-}CD83^{-}CD1a^{+}$), the cells were used in the assays. Cell viability was monitored using the CellTiter-Blue™ assay kit (Promega). None of the experimental treatments affected cell viability compared to medium-only control treatments. Human blood collections were conducted in compliance with established guidelines approved by the Institutional Review Board.

Mice

To generate BMDC, the bone marrow was obtained from wild-type or *Tlr2*$^{-/-}$ C57BL/6J mice (The Jackson Laboratory) as well as from *C3ar*$^{-/-}$, *C5ar1*$^{-/-}$ or *C5ar2*$^{-/-}$ (*C5L2*$^{-/-}$) mice, supplied from our colonies maintained at The Jackson Laboratory. The *C3ar*$^{-/-}$ mice were originally from Dr. Rick A. Wetsel (University of Texas) (Drouin *et al.*, 2002). The *C5ar1*$^{-/-}$ and *C5ar2*$^{-/-}$ were originally provided by Dr. Craig Gerard (Harvard Medical School) (Gerard *et al.*, 2005; Hopken *et al.*, 1996). All animal procedures were approved by the Institutional Animal Care and Use Committee and performed in compliance with established federal and state policies.

Intracellular survival assay

P. gingivalis ATCC 33277 was grown anaerobically from frozen stocks on modified Gifu anaerobic medium (GAM)-based blood agar plates for 5-6 days at 37°C, followed by anaerobic subculturing for 18-24 hours at 37°C in modified GAM broth containing 5 μg/ml hemin and 1 μg/ml menadione (Nissui Pharmaceutical). The viability of phagocytosed *P. gingivalis* was monitored by an antibiotic protection-based intracellular survival assay, essentially as we previously described (Wang *et al.*, 2007). Briefly, mammalian cells (BMDC, MDDC, or MDM) were allowed to phagocytose *P. gingivalis* (MOI = 10:1; $5x10^6$ bacteria and $5x10^5$ mammalian cells) for 30 min at 37°C. Extracellular nonadherent bacteria were removed by washing, while residual or extracellular adherent bacteria were killed by addition of gentamicin (300 μg/ml) and metronidazole (200 μg/ml) for 1 h. Immediately after, the cells were washed and lysed in sterile distilled water (20-min treatment at room temperature). Serial dilutions of the cell lysates were plated on blood agar plates and cultured anaerobically to determine viable counts (CFU) of internalized *P. gingivalis*. In certain experiments, the cells were incubated in the presence of C3a (200 nM), C5a (50 nM) and/or C5aR1A (1 μM), which was added 30 min prior to addition of Pg.

Flow cytometric internalization assay

Flow cytometry was used to measure phagocytosis of Pg as previously described (Wang *et al.*, 2007). Briefly, BMDC or peritoneal macrophages were incubated at 37°C with FITC-labeled Pg at a MOI of 10:1 for 30 min, at which time phagocytosis was stopped by cooling the incubation tubes on ice. After cell washing to remove nonadherent bacteria, extracellular fluorescence (representing attached but not internalized bacteria) was quenched with 0.2% trypan blue. The cells were washed again, fixed, and analyzed by flow cytometry (% positive cells for FITC-*P. gingivalis* and mean fluorescence intensity) using flow cytometry. Control experiments indicated that cytochalasin D-

pretreated cells incubated with FITC-*P. gingivalis* and subsequently exposed to trypan blue did not show significant fluorescence, thus confirming that cytochalasin D blocks internalization and that trypan blue quenches extracellular fluorescence. The phagocytosis index was calculated using the formula (% positive cells x MFI)/100.

Statistical analysis

Data were evaluated by analysis of variance and the Dunnett multiple-comparison test using the GraphPad Prism program, version 6.0h (GraphPad Software, San Diego, CA). Where appropriate (comparison of two groups only), two-tailed *t* tests were also performed. Statistical differences were considered significant at the level of $p < 0.05$. All experiments were performed at least twice for verification.

RESULTS

C5a enhances the intracellular killing of Pg by BMDC in a C5aR1-dependent way

Having established that Pg exploits C5aR1 to protect itself against killing by neutrophils and macrophages (Maekawa *et al.*, 2014; Wang *et al.*, 2010), we set out to determine whether this oral bacterium promotes its survival in DC by hijacking the same receptor. Using a standard intracellular survival assay that established the ability of Pg to persist within macrophages (Wang *et al.*, 2010; Wang *et al.*, 2007), we showed that viable Pg could be recovered from DC1.2 cells (**Fig. 1A**), a validated murine DC line (Shen *et al.*, 1997), consistent with earlier findings that Pg can survive within human myeloid DCs (Carrion *et al.*, 2012). Intriguingly, addition of C5a to the DC1.2 cells resulted in reduced Pg viable counts ($P < 0.01$; **Fig. 1A**), whereas the same treatment was previously shown to promote the intracellular survival of Pg in mouse macrophages (Wang *et al.*, 2010). The

inhibitory effect of C5a on Pg survival was abrogated when DC1.2 cells were pre-treated with an antagonist of C5aR1 (C5aR1A) (**Fig. 1A**). The ability of exogenously added C5a to promote the intracellular killing of Pg was confirmed using primary cells, specifically bone marrow-derived dendritic cells (BMDC) (**Fig. 1B**). In these experiments, we used BMDC from WT, *C5ar1*$^{-/-}$, and *Tlr2*$^{-/-}$ mice to better understand the involvement of these innate immune receptors in the handling of Pg by BMDC. Interestingly, Pg showed a decrease in intracellular viable counts in WT BMDC as compared to *C5ar1*$^{-/-}$ BMDC ($P < 0.01$; **Fig. 1B**), which is likely attributed to the endogenous synthesis of C5 and generation of C5a in BMDC cultures (Peng *et al.*, 2009) (hence, endogenous C5a can contribute to the killing of Pg in WT but not in in *C5ar1*$^{-/-}$ BMDC). In line with this finding, the antagonistic blockade of C5aR1 in WT BMDC promoted the intracellular survival of Pg ($P < 0.01$; **Fig. 1B**). As expected, treatments of *C5ar1*$^{-/-}$ BMDC with C5a and/or C5aR1A did not influence the Pg viable counts **(Fig. 1B)**, confirming that the effects of these treatments strictly require the presence of C5aR1 and do not involve possible off-target effects. Pg displayed increased survival also in *Tlr2*$^{-/-}$ BMDC (as compared to WT BMDC) ($P < 0.01$; **Fig. 1**). Therefore, in addition to C5aR, TLR2 also contributes to the intracellular killing of Pg in BMDC.

C5aR2 and C5aR1 differentially affect the intracellular survival of Pg in BMDC

We then addressed whether other complement anaphylatoxin receptors share the capacity of C5aR1 to contribute to the intracellular killing of Pg in BMDC. C5aR2 (also referred to as C5a-like receptor 2; GPR77) functions as an alternative high-affinity receptor for C5a (Monk *et al.*, 2007). In side-by-side experiments, using WT and *C5ar1*$^{-/-}$ BMDC as comparative controls, we found that the ability of Pg to survive intracellularly in *C5ar2*$^{-/-}$ BMDC was significantly reduced compared to WT BMDC, that is, C5aR2 deficiency had the exact opposite effect from that of C5aR1 deficiency **(Fig. 2)**. This finding is consistent with the notion that C5aR2 acts as a negative modulator of C5aR1 (Bamberg *et al.*, 2010), which would therefore be more active in *C5ar2*$^{-/-}$ BMDC to mediate Pg killing. As expected, the

intracellular survival of Pg in *C5ar2*$^{-/-}$ BMDC was not affected by the addition or not of exogenous C5a in the cultures **(Fig. 2)**.

C3a enhances the intracellular killing of Pg by BMDC a C3aR-dependent manner

In a further side-by-side experiment comparing the effect of C5aR1 with that of the C3a receptor (C3aR), we showed that – similarly to C5a – C3a also promoted the killing of Pg in WT BMDC (**Fig. 3**). C3a failed to modulate the killing of Pg in *C3ar*$^{-/-}$ BMDC (**Fig. 3**), thereby firmly establishing that the effect of C3a was specifically mediated by the C3aR.

The reduced intracellular viable counts of Pg in the presence of C3a or C5a might – at least in part – be attributed to decreased Pg phagocytosis by BMDC. This possibility was ruled out after we measured Pg phagocytosis by BMDC in the absence or presence of C3a or C5a. Indeed, none of the anaphylatoxins inhibited Pg phagocytosis but rather modestly- albeit significantly- promoted this function ($P < 0.01$) (**Fig. 4**). Therefore, C3a and C5a promote the phagocytosis and killing of Pg by BMDC.

C3a and C5a upregulate cytokine production in Pg-challenged BMDC

We have previously shown that C5a inhibits Pg-induced IL-12p70 in mouse macrophages, resulting in enhanced in vivo survival of Pg in the mouse host (Liang *et al.*, 2011). In view of the stimulatory effects of C5a (and C3a) on Pg killing in BMDC, we determined the effects of C5a and C3a on the induction of IL-12p70 in Pg-challenged BMDC. In contrast to the results obtained in macrophages, C5a enhanced Pg-induced IL-12p70 in BMDC (**Fig. 5**). C3a exerted a similar but less pronounced effect (**Fig. 5**). C5a also enhanced the production of IL-23, while C3a and C5a augmented the

production of TNF by Pg-challenged BMDC (**Fig. 5**), thereby firmly establishing that they exert stimulatory, and not immunosuppressive, effects in BMDC.

Differential effects of C5aR1 on Pg intracellular viability in human MDDC and MDM

We next examined whether the Pg killing-promoting effect of the C5a-C5aR1 axis is relevant to human myeloid dendritic cells. Consistent with the data in the mouse system (**Fig. 1**), C5a significantly enhanced the killing of Pg by human monocyte-derived dendritic cells (MDDC), whereas C5aR1A reversed this effect (**Fig. 6A**). In stark contrast, C5a enhanced the intracellular survival of Pg in human monocyte-derived macrophages (MDM) in a C5aR1-dependent manner (**Fig. 6B**), consistent with our earlier observations in mouse macrophages (Wang *et al.*, 2010). These data show that the cell-type specific effect of C5aR1 on Pg intercellular killing is not species-restricted in its mode of action.

Pg fails to utilize CR3 in BMDC

We have previously shown that Pg uses complement receptor 3 (CR3; CD11b/CD18) to enter and persist within mouse macrophages (Wang *et al.*, 2007). Although Pg failed to exploit C3aR or C5a1R in BMDC to enhance its survival, we set out to determine whether the observed intracellular viability of Pg could be attributed, at least in part, to exploitation of CR3 in BMDC. We first determined whether BDMC CR3 functions as a phagocytic receptor for Pg. However, a CR3 blocking mAb failed to inhibit the phagocytosis of Pg by BMDC (**Fig. 7A**), whereas the same mAb blocked the phagocytosis of Pg by mouse macrophages (**Fig. 7B**). Therefore, CR3 is not a phagocytic receptor for Pg in BMDC.

DISCUSSION

Our findings from this study show a context-dependent involvement of complement receptors in immune evasion by Pg. Although Pg can exploit C5aR1 in neutrophils and macrophages to undermine their antimicrobial function (Maekawa *et al.*, 2014; Wang *et al.*, 2010) as well as hijack CR3 for a safe entry into macrophages (Wang *et al.*, 2007), the same receptors on dendritic cells did not provide a survival advantage to Pg. In fact, C5aR1 facilitated the intracellular killing of Pg in dendritic cells, whereas CR3 did not function as a phagocytic receptor for Pg. Similar to C5aR1, C3aR enhanced the intracellular killing of Pg in dendritic cells, although in macrophages C3aR had no significant effect on the intracellular survival of Pg (Wang *et al.*, 2010). Thus, differences in signaling pathways between the various innate leukocytes may account for the distinct intracellular fate of Pg.

For instance, the diametrically opposed effects of C5aR1 in dendritic cells and macrophages (promoting intracellular killing of Pg in dendritic cells, but enhancing intracellular survival of Pg in macrophages) might be related to differential regulation of the cAMP response in these two cell types. In macrophages, activation of C5aR1 leads to increased levels of intracellular cAMP and hence PKA activation, which is critical for suppressing the nitric oxide-dependent killing of Pg (Wang *et al.*, 2010). Interestingly, C5aR1 signaling stimulates cAMP-dependent PKA activity also in neutrophils (Ward, 2004). In dendritic cells, however, C5aR1 was shown to inhibit cAMP production and thus the activation of PKA (Peng *et al.*, 2009). C3aR – which also promoted BMDC killing of Pg in this study – was also shown to inhibit the cAMP-PKA pathway, thereby lifting regulatory restraints on dendritic cell activation (Li *et al.*, 2008). Both C3aR and C5aR1 activate Gαi protein-mediated signaling. Upon Gαi activation, the released Giβγ subunits regulate the production of cAMP by adenylate cyclase, either in a positive or negative manner, depending on the enzyme isoform (Sunahara & Taussig, 2002). Interestingly, the adenylate cyclase isoforms that are positively regulated by Giβγ are distinct from those that are sensitive to the inhibitory action of Gαi (Sunahara & Taussig, 2002). Thus, it is

possible that dendritic cells and macrophages express different isoforms of adenylate cyclase, which in turn display differential regulation in response to C3aR- or C5aR-induced Gαi signaling.

Another cell type-specific difference we have observed in this study is that C5a promotes Pg-induced IL-12p70 in BMDC, whereas previously we have shown that the same ligand inhibits Pg-induced IL-12p70 in macrophages (Liang *et al.*, 2011). The C5a-induced inhibition of IL-12p70 by Pg was mediated by ERK1/2 signaling (Liang *et al.*, 2011), in line with earlier studies showing that C5a-induced ERK1/2 signaling inhibits enterobacterial lipopolysaccharide-induced IL-12p70 production (Hawlisch *et al.*, 2005). Whereas C5a was shown to induce ERK1/2 signaling also in dendritic cells (Weaver *et al.*, 2010), the ERK1/2 pathway in this cell type upregulates, rather than inhibits, IL-12p70 production (Baruah *et al.*, 2009).

C5aR2 is a relatively recently discovered C5a receptor, the function of which is largely enigmatic, if not controversial. Although originally perceived as a decoy receptor (Okinaga *et al.*, 2003), subsequent studies showed that C5aR2 interacts physically with and negatively regulates C5aR1 signaling, thereby having anti-inflammatory action (Bamberg *et al.*, 2010; Croker *et al.*, 2014; Gerard *et al.*, 2005). Yet, other studies have assigned a proinflammatory role for C5aR2 in various experimental systems and disease models (Pundir *et al.*, 2015; Rittirsch *et al.*, 2008; Selle *et al.*, 2015). Overall, it appears that the activities of C5aR2 are dynamic and contextual depending on cell type, tissue, and disease model (Li *et al.*, 2013). Our findings that C5aR1 promotes the intracellular killing of Pg in BMDC, whereas the absence of C5aR2 is associated with increased intracellular viable counts of Pg is consistent with the notion that C5aR2 acts as a negative regulator of C5aR1 (Bamberg *et al.*, 2010; Croker *et al.*, 2014).

Although Pg does not appear to exploit complement receptors (at least not those investigated here) to survive in dendritic cells, earlier work has shown that Pg can exploit other innate immune mechanisms to manipulate this cell type. Specifically, Pg uses its Mfa1 fimbriae to interact with a C-type lectin, the dendritic cell-specific ICAM-3 grabbing nonintegrin (DC-SIGN) (Zeituni *et al.*, 2009).

Through this interaction, Pg enters dendritic cells and promotes its survival while suppressing the maturation of the dendritic cells (Carrion *et al.*, 2012; Zeituni *et al.*, 2009). Interestingly, whereas DC-SIGN directs Pg into intracellular vesicles that escape early autophagosomal recognition and eventual autophagic degradation, TLR2 antagonizes autophagy evasion and thus the intracellular persistence of Pg (El-Awady *et al.*, 2015). Consistent with this finding, we found that that Pg exhibited increased viable counts in *Tlr2*$^{-/-}$ BMDC as compared to WT BMDC, thus indicating that TLR2 contributes to the intracellular killing of Pg. By using different fimbrial mutants of Pg that preferentially interact with DC-SIGN (Mfa1^{+}FimA^{-}) or TLR2 (Mfa1^{-}FimA^{+}), the same group showed that the Mfa1^{+}FimA^{-} strain displays increased intracellular survival than the WT strain, which in turn is more resistant to intracellular killing than the Mfa1^{-}FimA^{+} strain (El-Awady *et al.*, 2015). This earlier study in conjunction with our current findings suggest that DC-SIGN promotes the intracellular survival of Pg in dendritic cells, whereas TLR2 and complement receptors (C3aR and C5aR1) mediate the opposite effect, thus facilitating the clearance of Pg.

The association of the TLR2-interacting FimA fimbriae with increased intracellular killing in myeloid dendritic cells stands in stark contrast to the ability of the same fimbrial protein to mediate Pg evasion in macrophages (Wang *et al.*, 2007). In macrophages, activation of TLR2 by Pg leads to inside-out signaling that transactivates CR3 to adopt its high-affinity conformation (Harokopakis & Hajishengallis, 2005). Pg FimA fimbriae can then bind transactivated CR3 (through distinct FimA epitopes from those activating TLR2 (Hajishengallis *et al.*, 2005a)) leading to the internalization of Pg in a manner that avoids lysosomal degradation (Wang & Hajishengallis, 2008; Wang *et al.*, 2007). In contrast, Pg failed to utilize CR3 at in BMDC. The reason for this difference is not clear. However, as alluded to above, CR3 and other β2 integrins require transactivation via inside-out signaling to adopt their high-affinity conformation (Shimaoka *et al.*, 2002). In dendritic cells, β2 integrins including CR3 (CD11b/CD18; Mac-1) appear to be functionally inactive, as they cannot be readily activated by various physiologic stimuli (Varga *et al.*, 2007). CR3 activation could be achieved when supraphysiologic concentrations (5 mM) of $Mg2^{+}$ were used (Varga *et al.*, 2007). Our results here,

therefore, are consistent with and support this earlier observation (Varga *et al.*, 2007) and, taken together with our earlier findings (Wang *et al.*, 2007), show that Pg can transactivate and utilize CR3 in macrophages but not in dendritic cells.

In summary, complement receptors, such as C3aR, C5aR1, and CR3, mediate cell-type-specific effects on how innate leukocytes cope with Pg. Specifically, Pg exploits complement to promote its adaptive fitness in neutrophils and macrophages, but not in dendritic cells, where C3aR and C5aR1 actually facilitate the intracellular killing of this bacterium. These findings appear paradoxical given the fact that dendritic cells are not as potent in pathogen destruction as compared to neutrophils or macrophages (Silva). However, the immediate threat to Pg in its predominant niche, the periodontal pocket, is represented by neutrophils and secondarily by macrophages (these two cell types and especially the former predominate in the leukocyte infiltrate of the periodontal pocket (Delima & Van Dyke, 2003). Therefore, and given the abundance of complement activation products in the periodontal pocket (Hajishengallis, 2010), it makes evolutionary sense that Pg developed complement-dependent evasion mechanisms against the leukocyte types that are most often encountered in its niche.

Figure legends

Figure 1: C5a promotes the intracellular killing of Pg by mouse dendritic cells in a C5aR1-dependent manner. DC1.2 cells (**A**) or BMDC generated from WT, *C5ar1*$^{-/-}$, or *Tlr2*$^{-/-}$ mice (**B**) were incubated with Pg (MOI = 10:1) in the presence or absence of C5a (50 nM) and/or C5aR1A (1 μM). Using an antibiotic protection-based intracellular survival assay, viable counts of internalized bacteria at 90 min post-infection were determined by CFU enumeration. Data are means ± SD (*n*=3 sets of cell cultures). ***P* < 0.01 compared to medium-only control treatments or between indicated groups.

Figure 2: C5aR2 and C5aR1 mediate opposite effects on the intracellular survival of Pg in BMDC. BMDC generated from WT, *C5ar1*$^{-/-}$, or *C5ar2*$^{-/-}$ mice were incubated with Pg (MOI = 10:1) in the presence or absence of C5a (50 nM). Using an antibiotic protection-based intracellular survival assay, viable counts of internalized bacteria at 90 min post-infection were determined by CFU enumeration. Data are means ± SD (*n*=3 sets of cell cultures). ***P* < 0.01 compared to medium-only control treatments or between indicated groups.

Figure 3: C3a enhances the intracellular killing of Pg by BMDC a C3aR-dependent manner BMDC generated from WT, *C3ar*$^{-/-}$ (**A**) or *C5ar1*$^{-/-}$ (**B**) mice were incubated with Pg (MOI = 10:1) in the presence or absence of C3a (200 nM) or C5a (50 nM), respectively. Using an antibiotic protection-based intracellular survival assay, viable counts of internalized bacteria at 90 min post-infection were determined by CFU enumeration. The experiments were performed side-by-side and the medium-only treated WT group was common to both **A** and **B**. Data are means ± SD (*n*=3 sets of cell cultures). **P* < 0.05 and ***P* < 0.01 compared to medium-only control treatments.

Figure 4: C3a and C5a enhance the phagocytosis of Pg by BMDC. BMDC were incubated for 30 min with FITC-labeled Pg and phagocytosis was determined by flow cytometry after quenching extracellular fluorescence. The phagocytic index was calculated using the formula (% positive cells x MFI)/100. Data are means ± SD (*n*=3 sets of cell cultures). ***P* < 0.01 compared to medium-only control treatments.

Figure 5: C3a and C5a promote induction of proinflammatory cytokines in Pg-challenged BMDC. BMDC were incubated with medium only or with Pg (MOI = 10:1) and the levels of the indicated cytokines in culture supernatants, collected at 24h post-incubation, were determined by ELISA. Data

are means ± SD (*n*=3 sets of cell cultures). *P < 0.05 and **P < 0.01 compared to medium-only control treatments.

Figure 6: Differential effects of the C5a-C5aR1 axis on the intracellular survival of Pg in human MDDC vs. MDM. MDDC (**A**) or MDM were incubated with Pg (MOI = 10:1) in the presence or absence of C5a (50 nM) and/or C5aR1A (1 μM). Using an antibiotic protection-based intracellular survival assay, viable counts of internalized bacteria at 90 min post-infection were determined by CFU enumeration. Data are means ± SD (*n*=3 sets of cell cultures). **P < 0.01 compared to medium-only control treatments.

Figure 7: CR3 does not mediate Pg phagocytosis by BMDC. BMDC (**A**) or macrophages (**B**) were incubated for 30 min with FITC-labeled Pg, in the presence or absence of 10 μg/ml mAb to CD11b (CR3). Cytochalasin D (10 μg/ml) was used as positive control for phagocytosis inhibition in A. IgG2b was used as isotype control in B. Phagocytosis was determined by flow cytometry after quenching extracellular fluorescence. The phagocytic index was calculated using the formula (% positive cells x MFI)/100. Data are means ± SD (*n*=3 sets of cell cultures). **P < 0.01 compared to medium-only control treatments.

ACKNOWLEDGEMENTS

This work was supported by grants from the National Institutes of Health (DE015254 and DE021685 to G.H; AI068730 and AI030040 to J.D.L.).

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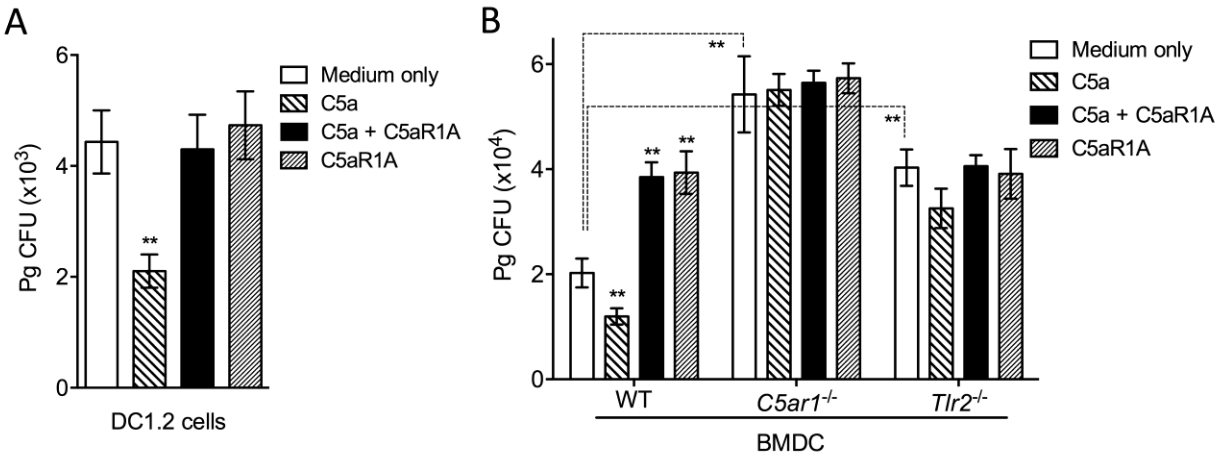
Figure 1



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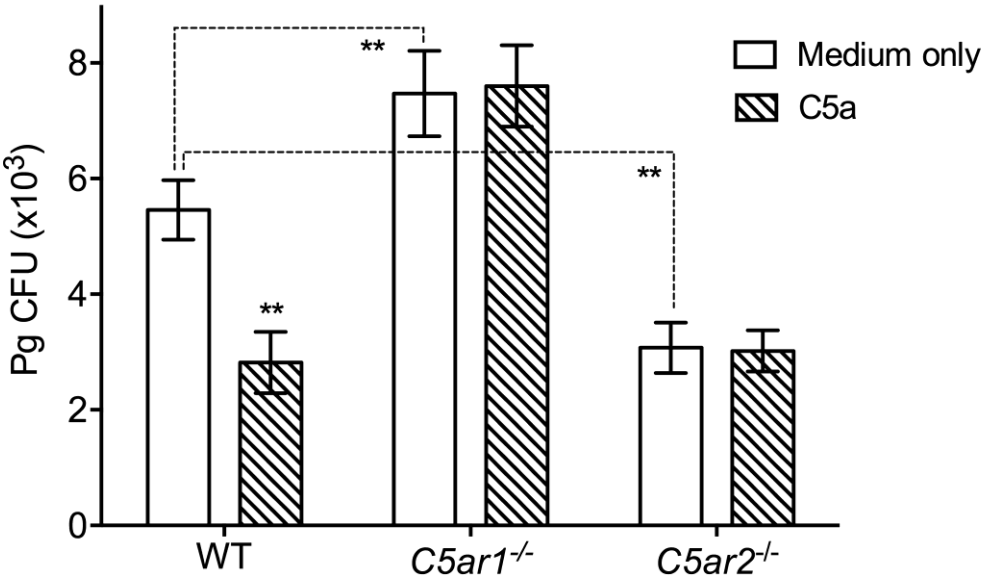

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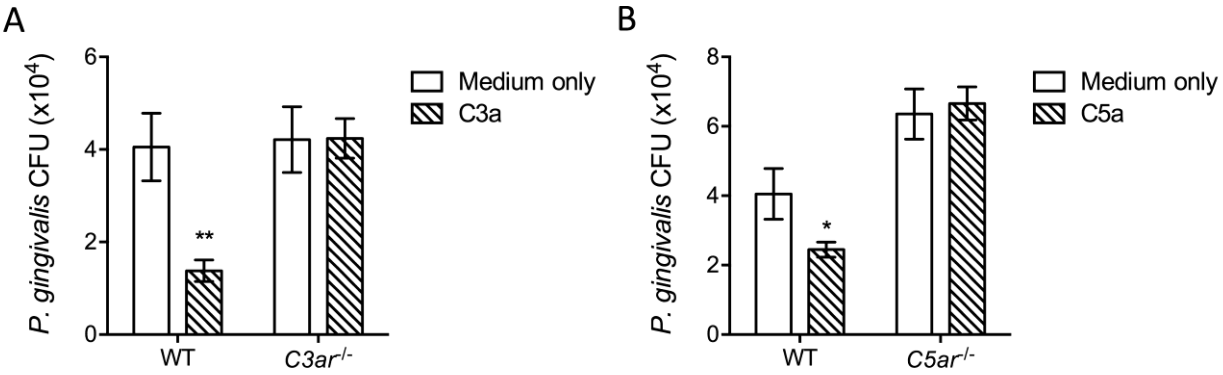

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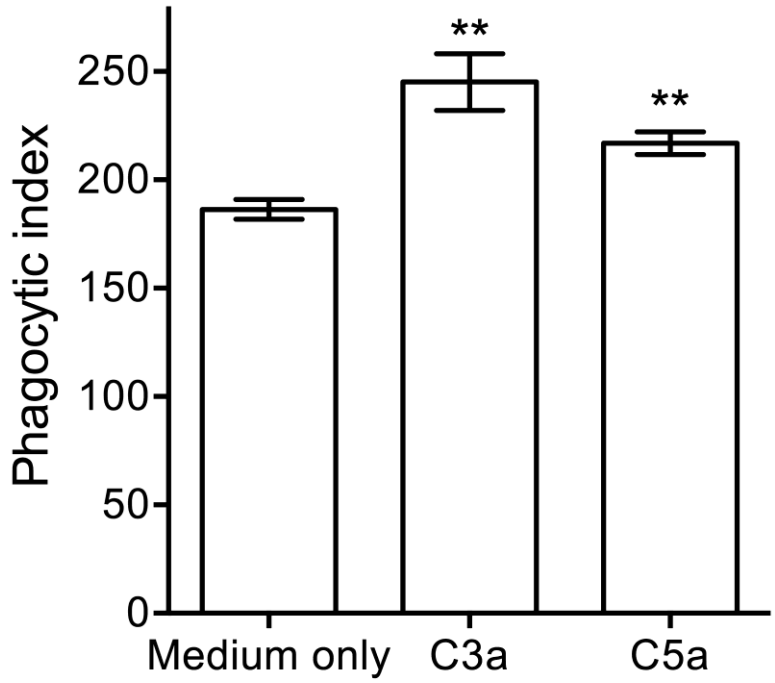

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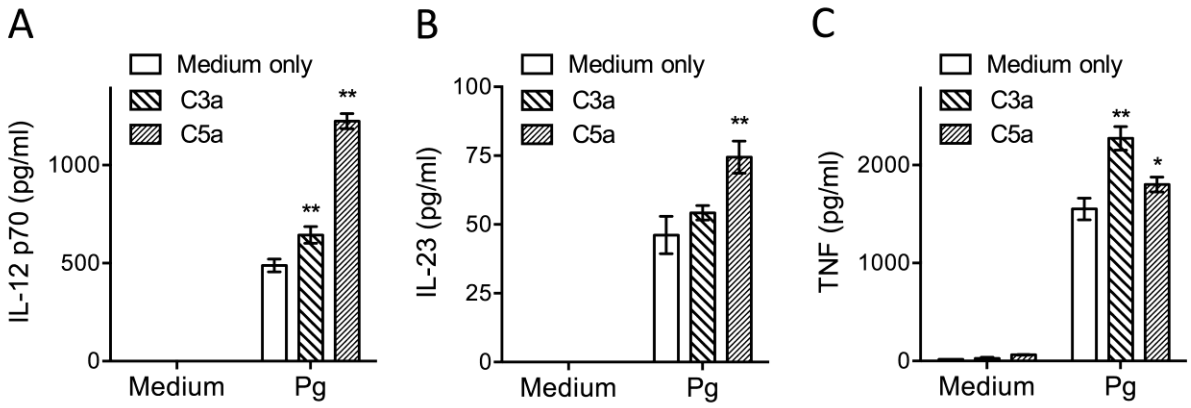

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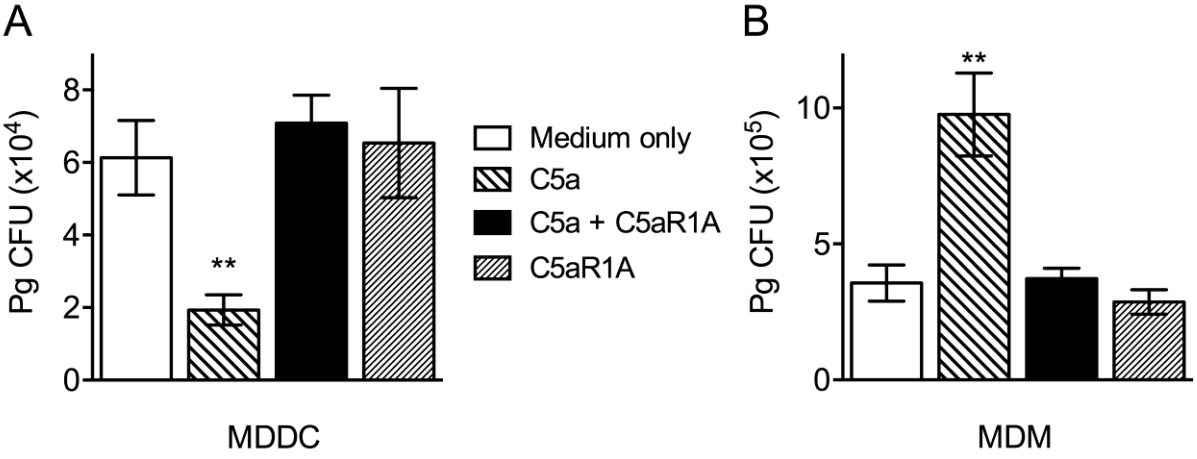

Figure 7