

Porphyromonas gingivalis Mediates Inflammasome Repression in Polymicrobial Cultures through a Novel Mechanism Involving Reduced Endocytosis*

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Background: *Porphyromonas gingivalis* has low immunogenicity and synergizes with other periodontal pathogens, including *Fusobacterium nucleatum*.

Results: *Porphyromonas gingivalis* selectively represses the activation of the IL-1 β -processing inflammasome by *Fusobacterium nucleatum* and inducers that are endocytosed.

Conclusion: *Porphyromonas gingivalis* suppresses inflammasome activity through a novel mechanism involving modulation of endocytosis.

Significance: Inflammasome suppression may contribute to periodontitis and other chronic diseases.

The interleukin (IL)-1 β -processing inflammasome has recently been identified as a target for pathogenic evasion of the inflammatory response by a number of bacteria and viruses. We postulated that the periodontal pathogen, *Porphyromonas gingivalis* may suppress the inflammasome as a mechanism for its low immunogenicity and pathogenic synergy with other, more highly immunogenic periodontal bacteria. Our results show that *P. gingivalis* lacks signaling capability for the activation of the inflammasome in mouse macrophages. Furthermore, *P. gingivalis* can suppress inflammasome activation by another periodontal bacterium, *Fusobacterium nucleatum*. This repression affects IL-1 β processing, as well as other inflammasome-mediated processes, including IL-18 processing and cell death, in both human and mouse macrophages. *F. nucleatum* activates IL-1 β processing through the Nlrp3 inflammasome; however, *P. gingivalis* repression is not mediated through reduced levels of inflammasome components. *P. gingivalis* can repress Nlrp3 inflammasome activation by *Escherichia coli*, and by danger-associated molecular patterns and pattern-associated molecular patterns that mediate activation through endocytosis. However, *P. gingivalis* does not suppress Nlrp3 inflammasome activation by ATP or nigericin. This suggests that *P. gingivalis* may preferentially suppress endocytic pathways toward inflammasome activation. To directly test whether *P. gingivalis* infection affects endocytosis, we assessed the uptake of fluorescent particles in the presence or absence of *P. gingivalis*. Our results show that

P. gingivalis limits both the number of cells taking up beads and the number of beads taken up for bead-positive cells. These results provide a novel mechanism of pathogen-mediated inflammasome inhibition through the suppression of endocytosis.

The innate immune inflammatory cytokine, interleukin (IL)-1 β is critical in the host defense against infection, and consequently, pathogens have evolved an array of mechanisms for inhibiting its production (1). This includes the inhibition of the transcription of the pro-IL-1 β precursor mRNA, the production of IL-1 β decoy receptors, and most recently, the inhibition of the IL-1 β processing inflammasome (see Ref. (2) for a review). The primary functions of the inflammasome are to cleave the precursor pro-IL-1 β protein to its active secreted form and to promote caspase-dependent signaling pathways leading to cell death in response to foreign or intrinsic “danger signals,” including bacterial-encoded “pattern-associated molecular patterns” and host-derived “danger-associated molecular patterns.” In addition to its role in combating pathogenic infection, recent evidence suggests a role for the inflammasome in mediating host metabolic responses (3, 4). The dysregulation of inflammasome components has been linked to a number of inherited inflammatory and immune disorders, further underscoring its relevance in human disease (5).

Classically, the inflammasome is composed of a nucleotide-binding domain and leucine-rich repeat (NLR)⁴ family protein, the adaptor molecule PYCARD/ASC, and pro-caspase-1, which provides the core enzymatic activity of the complex. Each of these components has been postulated to serve as a

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⁴ The abbreviations used are: NLR, nucleotide-binding domain and leucine-rich repeat; ASC, apoptosis-associated speck-like protein containing a CARD; PGN, peptidoglycan; BMDM, bone marrow-derived mouse macrophages; m.o.i., multiplicity of infection; MSU, monosodium urate.

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target for pathogenic inhibition by specific bacteria and viruses. At least two pathogenic viruses have evolved strategies for suppressing the inflammasome by producing inactivating homologs of apoptosis-associated speck-like protein containing a CARD (ASC) (6, 7). Other viruses have been shown to mediate inflammasome inhibition through their production of serpin family proteins (8–10) and others (11) that can directly interact with caspase-1. Additionally, a viral NLR homolog was recently described that can repress inflammasome activation by Kaposi sarcoma-associated herpes virus (12). An alternative strategy for pathogenic inflammasome inhibition involves the modulation of the upstream signals that lead to its activation. Several bacteria minimize the inflammasome response through the production of Type III secretion system effector molecules that either regulate caspase-1 activity (13–15) or mask inflammasome detection (16). *Staphylococcus aureus* suppresses the inflammasome through enzymatic modification of its cell wall peptidoglycan (PGN) to make it resistant to lysosomal degradation following endocytosis (17). A variety of additional bacterial proteins suppress the inflammasome by unknown mechanisms (18, 19).

Porphyromonas gingivalis is one of the most common pathogens in chronic periodontitis, and its presence in the oral cavity is associated with a variety of related systemic diseases (20). In addition to its immunostimulatory properties, evidence is emerging to suggest that *P. gingivalis* has evolved several distinct mechanisms for evasion of the immune response that may contribute to its ability to perpetuate the chronic state of periodontal diseases. Like other oral pathogens, *P. gingivalis* produces proteases and toxins that directly attack host tissue, as well as lipopolysaccharide (LPS) and other microbial products that induce inflammation. However, cell wall extracts and purified cellular components from *P. gingivalis* have comparatively weak host immunostimulatory activity (21–24). Additionally, *P. gingivalis* lipid A has a unique structure that can either stimulate or antagonize Toll-like receptor 4 (TLR4) activation depending on environmental growth conditions and its phosphorylation and acylation states (25–29). Furthermore, *P. gingivalis* actively limits the immune response by producing proteases that degrade complement component C3 and IgG (30) and promotes intrinsic signaling that inhibits E-selectin expression and secretion of the neutrophil chemoattractant IL-8 (31, 32). Additionally, *P. gingivalis* confers resistance to apoptosis induced by pharmacologic agents (33–35). It is likely that these evasion mechanisms may explain the pathogenic synergism that is observed between *P. gingivalis* and other periodontal bacteria in animal models of infection (36–39).

Given the knowledge that *P. gingivalis* has evolved a unique set of mechanisms for subverting inflammatory activity, we hypothesized that *P. gingivalis* may function to suppress the inflammasome. Our results demonstrate that *P. gingivalis* suppresses inflammasome activation by other pathogenic bacteria, and that this suppression occurs by a novel mechanism involving the blockade of endocytosis.

EXPERIMENTAL PROCEDURES

Mouse Strains and Macrophage Culture—*MyD88*^{-/-} mice were obtained from Dr. Shizuo Akira; *Nlrp3*^{-/-} mice from Mil-

lenium Inc.; *Asc*^{-/-} mice from Dr. Vishva Dixit at Genentech; and *Casp1*^{-/-} mice from Dr. Richard Flavell, Yale University. All the mice were backcrossed for a minimum of nine generations to C57BL/6 mice. Bone marrow-derived macrophages were harvested from WT or gene-deletion mice and cultured in DMEM 10% fetal calf serum, M-CSF for 6–7 days. Cells were plated without M-CSF 16 h prior to infection. Peripheral blood macrophages were isolated on buffy coats from healthy donors (American Red Cross). Primary human macrophages were harvested by Ficoll-Hypaque gradients and seeded in 10 ml of RPMI 1640 medium containing 10% heat-inactivated FBS. Nonadherent cells were removed prior to infection.

Bacterial Strains and Bacterial Infection—*P. gingivalis* strain A7436 was isolated from a refractory case of periodontitis and has been described previously (40). *P. gingivalis* strain 381, *F. nucleatum* strain PK 1594, and *Escherichia coli* strain LF82 were obtained from American Type Culture Collection (Manassas, VA). *P. gingivalis* and *F. nucleatum* were cultured anaerobically, and *E. coli* aerobically until late exponential phase (0.8–1.2 optical density units at 660 nm). Aliquots were stored in media containing 20% glycerol at -80 °C and used within 3–4 months of preparation. Bacterial counts were confirmed to within 2–3-fold by replating of frozen cultures. Infections were performed by adding the desired multiplicity of infection (m.o.i.) of *P. gingivalis* either alone or together with *F. nucleatum* or *E. coli* to macrophages for 16–18 h. 10 µg/ml gentamycin was added to cell cultures 2 h following infection.

Treatment with Signal 1 and Signal 2 Inducers—To induce signal 1 activation in macrophages, 1 µg/ml Ultrapure *E. coli* LPS (InvivoGen) was added to macrophage cultures for 3 h prior to the addition of the signal 2 activator. Signal 2 activation was induced by the addition of 2 mM ATP (Sigma) or 20 µM nigericin (InvivoGen) for 30 min; 200 µg/ml monosodium urate (MSU) or 400 µg/ml alum crystals (InvivoGen) for 6 h; or 20 µg/ml PGN from *S. aureus* (InvivoGen) for 14–16 h. Inhibition of second signal activation by *P. gingivalis* was assessed by adding bacteria immediately prior to the second signal inducer.

ELISA Analysis—Supernatants were collected 18–24 h following infection unless otherwise indicated. Secreted cytokine levels were assessed using the human ELISA set for IL-1β (BD Biosciences) or the human ELISA kit for IL-18 (R&D Systems). Samples were assayed within linear range.

RNA Isolation and Real-time PCR—RNA was isolated using RNeasy kits (Qiagen). Real-time PCR was performed using TaqMan® Assays on Demand (Applied Biosystems). Values represent averages ±S.D. of biological triplicates. All values were standardized to 18 S rRNA expression.

Western Analysis—For assessment of secreted IL-1β levels, supernatants were clarified by centrifugation for 10 min and then boiled for 5 min in 1/3 volume of 3× SDS sample buffer (187.5 mM Tris, pH 6.8, 6% SDS, 30% glycerol, 150 mM DTT, 0.03% bromophenol blue). Cell lysates were prepared by washing cells in 1× PBS and then lysing for 20 min in ice-cold 1× lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100) supplemented with Complete EDTA-free Protease Inhibitor (Roche Applied Science). Lysates were centrifuged for 10 min and boiled for 5 min in 1/3 volume of 3× SDS sample buffer. Immunoblots were processed using 3ZD

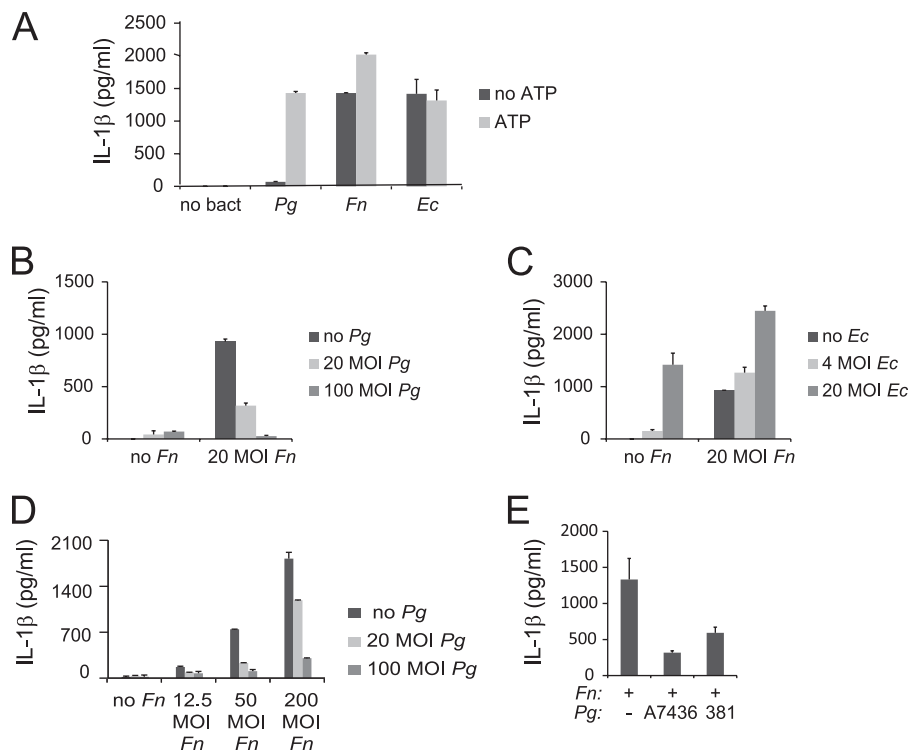


FIGURE 1. *P. gingivalis* lacks second signal activation ability for IL-1 β production and can repress second signal activation by *F. nucleatum*. ELISAs of IL-1 β in cell supernatants were performed following 12–16-h infection. A, mouse BMDM were infected with 20 m.o.i. *P. gingivalis* (*Pg*), *F. nucleatum* (*Fn*), or *E. coli* (*Ec*). Where indicated, cells were treated for 30 min with 2 mM ATP immediately prior to collection of supernatants. B, BMDM were infected with *Fn* and *Pg* alone or in combination as indicated. C, BMDM were infected with *Fn* and *Ec* alone or in combination as indicated. D, BMDM were treated with a range of doses of *Fn* and *Pg*. E, BMDM were treated with *Fn* and *Pg* strains A7436 or 381. Results represent the averages and standard deviation of duplicates and are representative of at least three independent experiments.

monoclonal antibody against Pro-IL-1 (Frederick National Laboratory for Cancer Research), H-153 against IL-1 β (Santa Cruz Biotechnology), IMG-5028 against caspase-1 (Imgenex), AL177 against ASC (Enzo Life Sciences), Clone Cryo-2 against NLRP3 (Enzo Life Sciences), sc-1615 against actin (Santa Cruz Biotechnology), and MAB374 against GAPDH (Millipore).

Cell Death Measurement—ToxiLight[®] bioassays were performed according to the manufacturer’s instructions (Lonza). Propidium iodide staining was performed as a measure of cell leakage upon death and Hoechst staining as a general nuclear stain as described previously (41).

Endocytosis Assays—FluoresbriteTN Carboxyl YG 2.0 Micron Microspheres latex beads (Polysciences, Inc.) were added to macrophages at a concentration of 10 beads/cell either alone or in combination with 100 moi *P. gingivalis*. 1 h following exposure, cells were washed three times with 1 \times PBS and then fixed with 4% paraformaldehyde. Cells and beads were visualized using a Zeiss 710 confocal microscope.

RESULTS

IL-1 β is produced in many circumstances via a two-step process involving the activation of transcription and translation of pro-IL-1 β (signal 1) followed by its proteolysis to an active secreted form by the inflammasome (signal 2). Most bacteria have the ability to provide both signals in macrophages, which are the most active physiologic producers of this potent inflammatory cytokine. By contrast, recent studies in epithelial cells suggest that *P. gingivalis* may lack the ability to produce a sec-

ond, inflammasome-activating signal (21). To test whether *P. gingivalis* lacks “signal 2” activation capability in macrophages, we measured IL-1 β production in bone marrow-derived mouse macrophages (BMDM) following infection. Our results showed that overnight infection with *P. gingivalis* was not sufficient for the stimulation of significant levels of IL-1 β , but that IL-1 β secretion could be induced by the subsequent addition of ATP, a well characterized second signal activator (42). This was in contrast to the high levels of IL-1 β activation elicited in the absence of ATP for *E. coli* and for *F. nucleatum*, another periodontal pathogen that is commonly isolated from periodontal disease sites together with *P. gingivalis* (20) (Fig. 1A). This suggests that the lack of a signal 2 activation capability is a property of *P. gingivalis* that is not conserved among all bacteria.

P. gingivalis has been shown to promote synergistic pathogenicity in polymicrobial infections with *F. nucleatum* (36–39). Given the emerging role of inflammasome repression as a mechanism for pathogenic stealth (2), we postulated that *P. gingivalis* may contribute to the synergistic effects of these two bacteria *in vivo* by repressing *F. nucleatum*-mediated activation of IL-1 β processing. To assess this as a possible mechanism, IL-1 β levels were measured following infection with 20 m.o.i. *F. nucleatum* and increasing doses of *P. gingivalis*. Results verified that *P. gingivalis* repressed IL-1 β activation by *F. nucleatum* (Fig. 1B). This mechanism of *P. gingivalis* in repressing *F. nucleatum* is not a common feature shared with other bacte-

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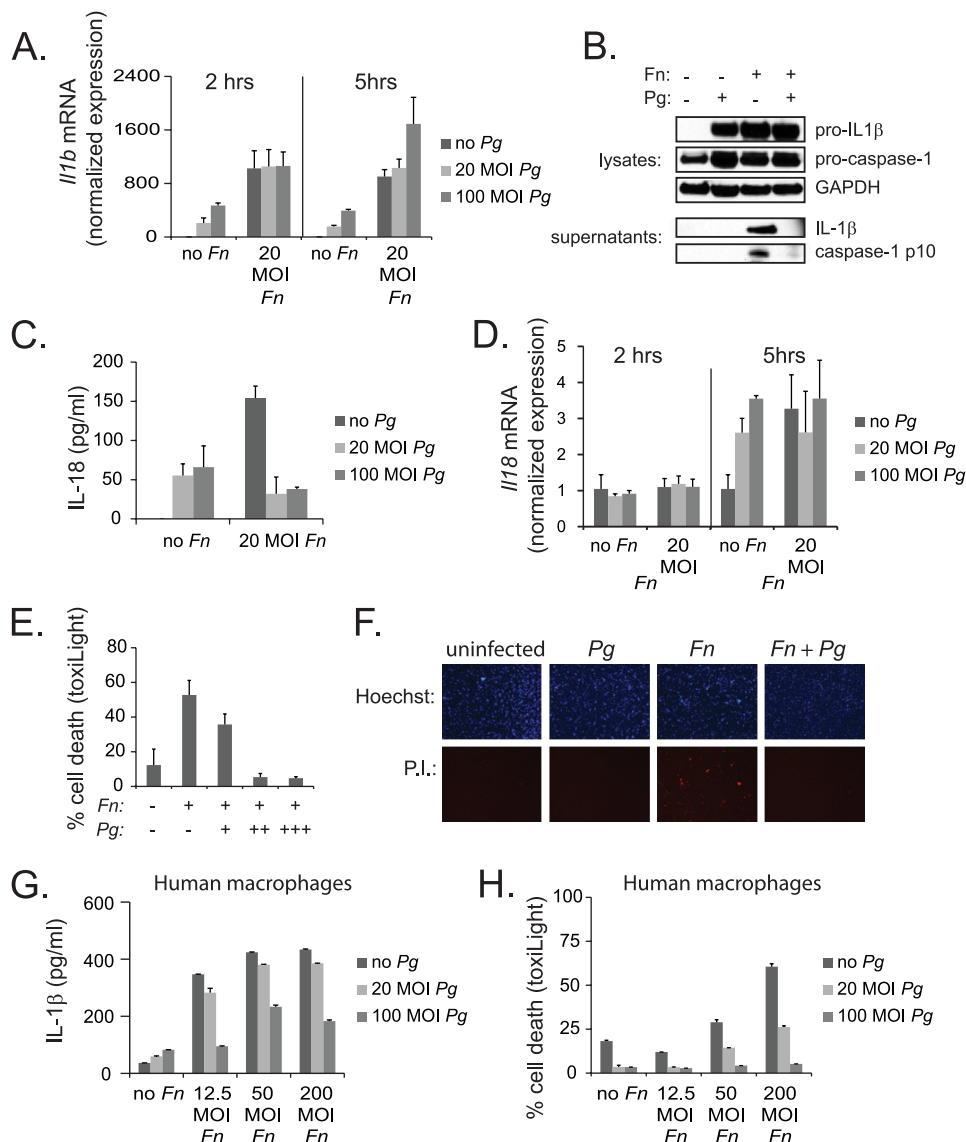


FIGURE 2. *Pg* represses inflammasome activation by *Fn*. *A*, real-time PCR of *Il1b* mRNA in mouse BMDM was performed 2 or 5 h following infection with a range of doses of *Fn* and *Pg*. Expression is normalized to the expression of 18 S rRNA and standardized to 1 in uninfected cells. Results represent the average \pm S.D. (error bars) of biological triplicates and are representative of three independent experiments. *B*, Western analysis is shown for pro-IL-1 β and pro-caspase-1 protein in cell lysates and cleaved (activated) IL-1 β and caspase-1 p10 in cell supernatants 14 h following infection with 20 m.o.i. *Fn* and 100 m.o.i. *Pg*. GAPDH is shown as a loading control. Results are representative of three independent experiments. *C*, ELISA of IL-18 expression in BMDM is shown following 14-h infection with *Fn* and *Pg* at indicated m.o.i. Results represent the averages \pm S.D. of duplicates and are representative of at least three independent experiments. *D*, real-time PCR of *Il18* mRNA in BMDM was performed 2 or 5 h following infection with a range of dose of *Fn* and *Pg*. Expression is normalized to the expression of 18 S rRNA and standardized to 1 in uninfected cells. Results represent the averages \pm S.D. of biological triplicates and are representative of three independent experiments. *E*, percentage of cell death in BMDM was determined by ToxiLight[®] assay 16 h following infection with 20 m.o.i. *Fn* alone or together with (+) 12.5, (++) 50, or (+++) 200 m.o.i. *Pg*. Results are representative of three independent experiments. *F*, propidium iodide (P. I.) stain is shown as an indicator of cell death in BMDM 16 h following infection with 20 m.o.i. *Fn* and 100 m.o.i. *Pg*. Hoechst staining is shown as a control. Results are representative of two independent experiments. *G*, ELISA of IL-1 β secretion in human macrophages is shown 14 h following infection with *Fn* and *Pg* at a range of m.o.i. Results represent the averages \pm S.D. of duplicates and are representative of two independent experiments. *H*, percentage of cell death in human macrophages was determined by ToxiLight[®] assay 16 h following infection with *Fn* and *Pg* at a range of m.o.i.

ria, given that *E. coli*, when added to *F. nucleatum*, enhanced, rather than repressed IL-1 β release (Fig. 1C). The repressive effect of *P. gingivalis* is dose dependent at a range of m.o.i. of *F. nucleatum* (Fig. 1D). Furthermore, this activity does not appear to be strain-specific, given that both the A7436 and 381 strains of *P. gingivalis* were able to repress *F. nucleatum*-mediated IL-1 β activation (Fig. 1E).

To distinguish the level at which *P. gingivalis* represses *F. nucleatum*-mediated IL-1 β activation, RNA was isolated from macrophages following infection with *F. nucleatum* alone

or in combination with *P. gingivalis*. Real-time PCR analysis of *Il1b* levels showed that this gene was transcriptionally induced by *F. nucleatum*, but that its induction was not repressed upon co-infection with *P. gingivalis* (Fig. 2A). These results indicate that *P. gingivalis* repression was not at the level of the activation of transcription (signal 1), implying a role in the IL-1 β processing pathway. To determine whether *P. gingivalis* affects inflammasome activation by *F. nucleatum* (signal 2), levels of pro-IL-1 β and pro-caspase-1 in cell lysates and levels of mature IL-1 β and caspase-1 p10 in supernatants were examined by

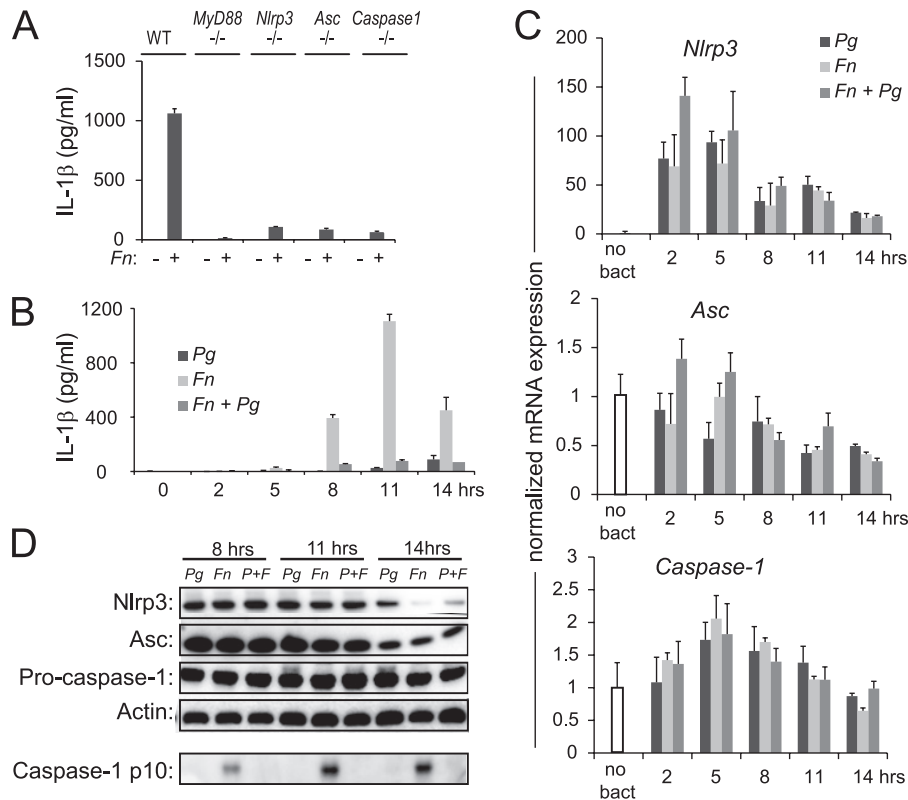


FIGURE 3. *Pg* does not repress the inflammasome by ablating the expression of its protein components. *A*, ELISA of IL-1 β secretion is shown for BMDM isolated from wild type (WT) and gene-deletion mice 16 h following infection with *Fn*. *B*, ELISA of IL-1 β secretion in wild type mouse BMDM is shown following a time course of infection with 20 m.o.i. *Fn* and 100 m.o.i. *Pg* alone or in combination. *C*, real-time PCR of *Nlrp3*, *Asc*, and *caspase-1* mRNA in BMDM following a time course of infection with 20 m.o.i. *Fn* and 100 m.o.i. *Pg* is shown. Expression is normalized to the expression of 18 S rRNA and standardized to 1 in uninfected cells. Results represent the average \pm S.D. (error bars) of biological triplicates and are representative of three independent experiments. *D*, Western analysis was performed for Nlrp3, Asc, and caspase-1 protein levels in BMDM 8, 11, and 14 h following infection with 20 m.o.i. *Fn* and 100 m.o.i. *Pg*. Actin is shown as a loading control, and cleaved caspase-1 in cell supernatants is provided for reference. Results are representative of three independent experiments.

Western analysis. Whereas the pro-forms of each protein were expressed in *F. nucleatum*-infected cells both without and with *P. gingivalis* co-infection, the mature forms were only detected in cells infected with *F. nucleatum* alone (Fig. 2*B*). These results verify that *P. gingivalis* blocks IL-1 β secretion at the level of inflammasome activation.

The inflammasome is responsible for the processing and secretion of other inflammatory cytokines besides IL-1 β , including IL-18, as well as for the promotion of cell death. Our results showed that, consistent with its role in inflammasome suppression, *P. gingivalis* repressed the secretion of IL-18 (Fig. 2*C*), and that this repression, like that of IL-1 β , did not appear to be transcriptional (Fig. 2*D*). *P. gingivalis* also suppressed the induction of cell death by *F. nucleatum* as measured by Toxi-Light[®] assay (Fig. 2*E*) or propidium iodide staining (Fig. 2*F*). These findings rule out the possibility that reduced cytokine secretion was caused by *P. gingivalis*-induced cell death in *F. nucleatum*- and *P. gingivalis*-infected cells and further support a role for *P. gingivalis* in inhibiting multiple signaling pathways downstream of the inflammasome. *P. gingivalis* was also able to repress IL-1 β levels and cell death in human macrophages, demonstrating that these findings are not limited to murine cells (Fig. 2, *G* and *H*).

One potential mechanism of inflammasome repression involves the modulation of the expression of inflammasome components. The most commonly utilized pathway of inflam-

masome activation requires an initial activation through the MyD88 adaptor molecule, followed by the assembly of a protein complex composed of NLRP3, ASC, and caspase-1 (43). To determine whether MyD88 and these three inflammasome components are required for *F. nucleatum* activation, we infected macrophages from gene-deletion mice with *F. nucleatum* and measured IL-1 β release. Our results showed that each of these genes was required for *F. nucleatum*-mediated IL-1 β release (Fig. 3*A*). We next tested whether transcript levels of the three inflammasome components were regulated by *F. nucleatum* and *P. gingivalis*. RNA was isolated over a time course of infection that preceded and spanned the 11-h peak of IL-1 β induction following *F. nucleatum* infection (Fig. 3*B*). *Nlrp3* transcription was activated by both *P. gingivalis* and *F. nucleatum*, and levels persisted following infection with the two bacteria in combination at each time point (Fig. 3*C*, top panel). By contrast, levels of *Asc* and *caspase-1* were not significantly modulated following infection with *F. nucleatum* either alone or in combination with *P. gingivalis* throughout the time course (Fig. 3*C*, bottom two panels). To determine whether repression of these inflammasome components occurs at the level of protein expression, Western blotting was performed 8, 11, or 14 h post-infection. Levels of Nlrp3, Asc, and pro-caspase-1 were similar in lysates from cells infected with *F. nucleatum* alone or in combination with *P. gingivalis* at each earlier time point. At the 14-h time point, levels of Nlrp3, Asc, and pro-caspase-1 in

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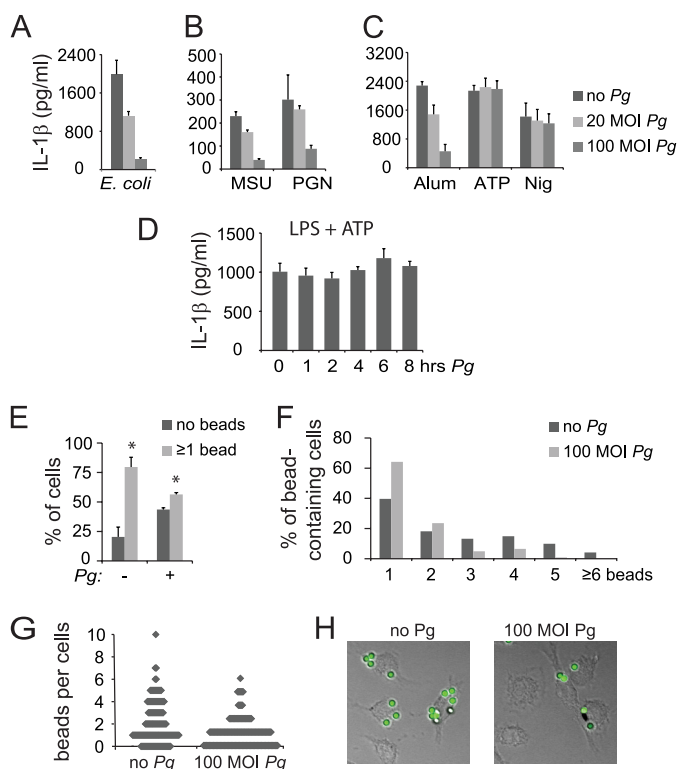


FIGURE 4. *Pg* inflammasome repression is mediated through blockade of endocytosis. ELISA of mouse BMDM demonstrates that *Pg* can repress the activation of IL-1 β by (A) *E. coli*; by (B) LPS plus MSU or PGN; and by (C) LPS plus alum, but not LPS plus ATP or nigericin (*Nig*). Results are representative of three independent experiments. D, IL-1 β ELISA of BMDM treated with LPS plus ATP is shown. *Pg* was added over a time course preceding the ATP treatment. Representative of two independent experiments. E, percentages of BMDM uptaking beads following 2-h exposure are shown. Cells were pre-treated with 100 m.o.i. *Pg* where indicated. F, numbers of beads in the bead-containing cell population for uninfected cells and cells infected with 100 m.o.i. *Pg* are shown. Numbers are expressed as a percentage of the total bead-containing cells. G, a diagrammatic representation of the number of beads per cell for uninfected cells and cells infected with 100 m.o.i. *Pg* is shown. Each cell is represented as a dot. H, a representative confocal microscopy image for bead uptake in uninfected cells and cells infected with 100 m.o.i. *Pg* is provided. The number of internalized beads was counted in >100 cells per each of three independent experiments.

P. gingivalis-infected cells were similar or higher than *F. nucleatum*- and *P. gingivalis*+*F. nucleatum*-infected cells, though the processing of pro-caspase-1 to caspase-1 only occurred in *F. nucleatum*-infected cells and not the other groups. These findings clearly demonstrate that *P. gingivalis* does not suppress inflammasome activation by reducing the expression of the inflammasome components (Fig. 3D).

To determine whether *P. gingivalis*-mediated inhibition is limited to *F. nucleatum* activation of the inflammasome, we tested whether *P. gingivalis* inhibits *E. coli* as an alternate bacterial NLRP3 inflammasome inducer. Results showed that *P. gingivalis* also can inhibit *E. coli*-mediated IL-1 β release (Fig. 4A). To further delineate the mechanism of *P. gingivalis* inflammasome inhibition, we tested the effect of *P. gingivalis* infection upon stimulation with LPS plus a panel of signal 2 inducers that are known to activate the NLRP3 inflammasome, including MSU, PGN, alum, ATP, and nigericin. Interestingly, *P. gingivalis* inhibited activation by MSU, PGN, and alum but did not inhibit activation by ATP and nigericin under the same conditions of *P. gingivalis* addition (Fig. 4, B and C). One difference

between ATP/nigericin and the other NLRP3 inflammasome inducers is the shortened time required for IL-1 β stimulation. For inflammasome stimulation by ATP and nigericin, cells are treated with LPS for 3 h and then with ATP for 30 min. By contrast, to achieve optimal inflammasome activation, other inducers are applied for 6 h to overnight. To determine whether a critical time of *P. gingivalis* exposure is required for its inhibition of ATP-mediated inflammasome activation, *P. gingivalis* was applied at increasing times prior to the activation of the inflammasome by ATP. However, prolonged *P. gingivalis* exposure did not restore its ability to repress inflammasome activation, suggesting that its failure to repress ATP-mediated inflammasome activation is not explained by differential timing (Fig. 4D).

An additional difference between ATP and nigericin and the other NLRP3 inflammasome inducers is the requirement for endocytosis to elicit IL-1 β production. The inducers that were blocked by *P. gingivalis* all require endocytosis to activate the signal; however, ATP and nigericin are thought to activate the inflammasome directly (43). One possible explanation for the selective ability of *P. gingivalis* to block IL-1 β production could be that *P. gingivalis* has the ability to regulate endocytosis. To determine whether this might be the case, we exposed macrophages to nonimmunogenic fluorescent latex beads and examined uptake by confocal microscopy 1 h following incubation. Our data showed that ~80% of the uninfected cells took up one or more beads, with ~20% of the cells having no bead uptake; however, cells that were infected with *P. gingivalis* prior to exposure to beads had a distribution of bead-positive and -negative cells closer to 50:50 (Fig. 4E). Furthermore, among the bead-positive cells, *P. gingivalis*-infected cells were more likely to contain one or two beads, whereas the uninfected cells were more likely to contain three or more beads (Fig. 4, F–H). These findings suggest that *P. gingivalis* infection causes reduction in levels of endocytosis.

DISCUSSION

P. gingivalis resides within periodontal tissue in combination with a wide variety of other pathogenic bacteria that can mediate a robust immune response, chief among which is *F. nucleatum*. *In vivo* studies have suggested that *P. gingivalis* and *F. nucleatum* can work synergistically to promote infection and pathogenesis (36–39). The mechanism for *F. nucleatum*-mediated synergy is postulated to involve its abundant production of adhesins that facilitate the co-aggregation of bacteria and attachment to host cells (44). However, the contribution of *P. gingivalis* to this synergy has remained largely unknown. We postulated that *P. gingivalis* may contribute to the synergy between the two bacteria through the evasion of *F. nucleatum*-mediated immune responses by inflammasome suppression. Inflammasome suppression is employed as a stealth mechanism by a variety of other pathogenic viruses and bacteria (2) and would be a likely avenue for promoting the chronic state of periodontitis through immune evasion. Furthermore, *P. gingivalis* has intrinsically low ability to stimulate immune responses (21–24), which could contribute to its stealth potential. Given these considerations, we sought to specify the effects of *P. gingivalis* on inflammasome activation in macrophages and to

determine whether *P. gingivalis* has the ability to actively suppress inflammasome induction by *F. nucleatum* and other stimulatory agents.

Inflammasome activation is a two-step process, and generalized low stimulatory responses to *P. gingivalis* may be explained in part by our findings that *P. gingivalis* can induce the transcription of the IL-1 β gene (signal 1) but that it lacks the ability to activate the processing of mature IL-1 β (signal 2) in primary mouse macrophages. The ability of *P. gingivalis* to stimulate first signal activation without second signal activation is unique among pathogenic bacteria (Ref. 5 and Fig. 1). Additionally, this selective first signal activation could explain why *P. gingivalis* has the ability to stimulate IL-1 β and cell death in monocytic cells (45–47), but not macrophages (Figs. 1 and 2). Immature monocytes are able to provide an endogenous signal that negates the requirement for second signal activation, but this endogenous second signal is lost upon maturation (48, 49).

Our results show that *P. gingivalis* can actively suppress the secretion of IL-1 β induced by *F. nucleatum*. Given that co-infection with *P. gingivalis* blocks the *F. nucleatum*-mediated activation of caspase-1 without reducing *F. nucleatum*-induced IL-1 β transcript levels, the inflammasome suppression by *P. gingivalis* occurs at the level of the second signal. These results are supported by the finding that *P. gingivalis* also can repress the secretion of IL-18, another cytokine that is known to be processed by the inflammasome, without affecting its transcript levels. Suppression is not explained by death induction by *P. gingivalis*. Instead, *P. gingivalis* is shown to suppress *F. nucleatum*-induced cell death. Cell death is an additional physiological process that is associated with inflammasome activation, and its suppression, therefore, is consistent with the notion that *P. gingivalis* IL-1 β suppression is an inflammasome-mediated event. The induction of IL-1 β and cell death also is suppressed in primary human macrophages, further supporting the findings that *P. gingivalis* acts at the level of the second signal. The suppression of the innate immune response by *P. gingivalis* in human macrophages could facilitate the maintenance of the chronic state of infection during periodontal diseases.

We propose a novel mechanism for *P. gingivalis*-mediated inflammasome inhibition through the suppression of endocytosis, as is indicated by the selectivity of *P. gingivalis* in its ability to inhibit Nlrp3 inflammasome inducers that are endocytosed. As a direct measure of effects on endocytosis, *P. gingivalis* infection was shown to modulate the levels of endocytosis of nonimmunogenic fluorescent beads. Additionally, our results rule out the possibility that *P. gingivalis* suppresses *F. nucleatum*-induced inflammasome activation through the reduction of endogenous levels of Nlrp3 or the other core components of its inflammasome. A recent study suggested that Nlrp3 is down-regulated by 33% in gingival fibroblast cultures colonized with a nine-species biofilm upon the inclusion of *P. gingivalis* (50). However, *IL1b* transcription was also reduced 35%, and it is therefore unclear whether reduced *IL1b*, rather than *Nlrp3*, may contribute to the reduced IL-1 β secretion in that model. *P. gingivalis* can activate *Nlrp3* transcription both in primary macrophages (Fig. 3) and in primary human gingival cells (51). Additionally, *P. gingivalis* does not significantly alter ASC or

pro-caspase-1 levels. Therefore, the ability of *P. gingivalis* to suppress IL-1 β in our model system is not likely to involve reduction in components of the core Nlrp3 inflammasome, but rather its ability to suppress endocytosis.

Studies to determine which components of *F. nucleatum* require phagocytosis to activate the inflammasome may help to further characterize mechanisms of *P. gingivalis*-mediated repression of endocytosis. There are multiple steps in the cellular pathway toward endocytosis, and it would be of interest to identify the step or steps that are affected. Given that *P. gingivalis* has been shown to cause actin cytoskeletal rearrangements (52), it is intriguing to postulate that *P. gingivalis* might suppress endocytosis by modulating the function of a core cytoskeletal protein. *P. gingivalis* has been shown to evade the endocytic pathway to lysosomes in favor of trafficking to autophagosomes (53), where it activates autophagy to provide a replicative niche (54); thus, there is a basis for its role in directing cellular trafficking.

An additional important future direction would include the identification of a *P. gingivalis* component responsible for inflammasome inhibition through the suppression of endocytosis. Interestingly, *S. aureus* produces an enzyme that suppresses its own inflammasome activation by making the cell wall PGN-resistant to degradation during phagocytosis (17). Although *P. gingivalis* produces abundant gingival proteases, a similar mechanism for enzymatic modulation of bacterial immunostimulatory components has not been determined. Our unpublished results suggest that *P. gingivalis* LPS is not sufficient for inflammasome inhibition, but that another secreted component may be involved.⁵ The identification and purification of this component could lead to the development of a therapeutic agent for the treatment of periodontitis, and considering that *P. gingivalis* can suppress the activation of the inflammasome by MSU and other danger-associated molecular patterns and pattern-associated molecular patterns, its therapeutic value could potentially extend to the treatment of gout and other inflammatory disorders.

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⁵ D. J. Taxman, unpublished data.

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