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***Filifactor alocis* modulates human neutrophil antimicrobial functional responses**

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Summary

Filifactor alocis is a newly appreciated pathogen in periodontal diseases. Neutrophils are the predominant innate immune cell in the gingival crevice. In this study we examined modulation of human neutrophil antimicrobial functions by *F. alocis*. Both non-opsonized and serum opsonized *F. alocis* were engulfed by neutrophils, but were not efficiently eliminated. Challenge of neutrophils with either non-opsonized or serum opsonized *F. alocis* induced a minimal intracellular as well as extracellular respiratory burst response compared to opsonized *Staphylococcus aureus* and fMLF, respectively. However, pre-treatment or simultaneous challenge of neutrophils with *F. alocis* did not affect the subsequent oxidative response to a particulate stimulus, suggesting that the inability to trigger the respiratory response was only localized to *F. alocis* phagosomes. In addition, while neutrophils engulfed live or heat-killed *F. alocis* with the same efficiency, heat-killed *F. alocis* elicited a higher intracellular respiratory burst response compared to viable organisms, along with decreased surface expression of CD35, a marker of secretory vesicles. *F. alocis* phagosomes remained immature by delayed and reduced recruitment of specific and azurophil granules, respectively. These results suggest that *F. alocis* withstands neutrophil antimicrobial responses by preventing intracellular ROS production, along with specific and azurophil granule recruitment to the bacterial phagosome.

Introduction

Periodontal disease is a polymicrobial induced disease initiated by the accumulation of pathogenic bacterial communities in the gingival crevice (Hajishengallis & Lamont, 2016). In the polymicrobial synergy and dysbiosis (PSD) model of the etiology and pathogenesis of

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periodontitis, synergistic interactions among community inhabitants increase the pathogenic potential, or nososymbiocity, of the community, and induce dysbiotic host responses that fail to control the bacterial challenge and, moreover, contribute to tissue destruction (Hajishengallis, 2013; Hajishengallis & Lamont, 2012; Lamont & Hajishengallis, 2015). The PSD model indicates that nososymbiotic communities incite a cyclic inflammation that clears beneficial bacteria while facilitating the growth of the pathogens (Hajishengallis & Lamont, 2012). It is estimated that over 40% of the adult population of the US will experience some form of periodontal disease (Eke, Dye, Wei, Slade, Thornton-Evans, Borgnakke, ... Genco, 2015). This disease has also been associated with several comorbidities including rheumatoid arthritis and cardiovascular disease among other inflammatory conditions (Bingham & Moni, 2013; Douglass, 2006; Kebschull, Demmer, & Papapanou, 2010; Kumar, 2013; Maddi & Scannapieco, 2013).

Studies of the etiology of periodontal disease have historically focused to a large extent on a limited number of cultivable bacteria which are associated with the initiation and progression of disease. Recent microbiome studies, however, have identified a large number of additional organisms that also exhibit a strong correlation with disease. One such species is *Filifactor alocis*, a gram-positive, rod-shaped, slow-growing asaccharolytic organism (Aruni, Chioma, & Fletcher, 2014). *F. alocis* shares common characteristics with other periodontal pathogens such as a resistance to oxidative stress, evasion of host immune system, biofilm formation and secretion of proteases (Aruni, Roy, & Fletcher, 2011; Aruni, Chioma, & Fletcher, 2014). Additionally, in multispecies communities, *F. alocis* can facilitate the growth of *Porphyromonas gingivalis* (Wang, Wright, Dingming, Uriarte, & Lamont, 2013), a keystone periodontal pathogen (Hajishengallis, Darveau, & Curtis, 2012). The ability of *F. alocis* to cause disease may not be solely restricted to the periodontal pocket. Mouse models have also shown the *F. alocis* can spread to other tissue organs such as spleen, lung and kidney tissues with acute kidney damage resulting from inflammation (Wang, Jotwani, Le, Krauss, Potempa, Coventry, ... Lamont, 2014).

The first immune cell to respond and be recruited in high numbers to the periodontal site is the neutrophil (Ryder, 2010; Scott & Krauss, 2012; Uriarte, Edmisson, & Jimenez-Flores, 2016). However, neutrophils largely fail to control the microbial infection, leading to a break of the homeostatic balance, and perpetuation of a chronic inflammatory environment which benefits inflammophilic organisms (Hajishengallis, 2014). Neutrophils possess numerous strategies to locate, detain and kill microbes (Amulic, Cazalet, Hayes, Metzler, & Zychlinsky, 2012; Kolaczowska & Kubes, 2013). One of the most critical anti-microbial mechanisms is the induction of the oxygen-dependent or respiratory burst response, where activation of the NADPH oxidase complex results in high and rapid oxygen consumption and generation of oxygen radicals (Nauseef, 2007). In addition, maturation of the phagosome is achieved by the recruitment of neutrophil granules to the bacteria-phagosome, representing an oxygen-independent neutrophil antimicrobial mechanism (Nordenfelt & Tapper, 2011).

In the present study, we demonstrate that human neutrophils can efficiently phagocytize both non-opsonized and opsonized *F. alocis*, that the oral pathogen can survive over 6 hours inside neutrophils which mount a minimal respiratory burst response, and that the majority

of *F. alocis* phagosomes are devoid of both specific and azurophil granules. The minimal respiratory burst response induced by *F. alocis* together with the low percent of phagosomes enriched with both specific granules and azurophil granules supports the hypothesis that this oral pathogen can manipulate neutrophil killing mechanisms to promote survival.

Results

Efficient phagocytosis of *F. alocis* by human neutrophils

Neutrophils respond to the presence of bacteria in the gingival compartment by exiting the blood vessels and migrating into the gingival crevice (Scott & Krauss, 2012). Phagocytosis is the first mechanism neutrophils utilize to neutralize bacterial challenge. Imaging flow cytometry was used to determine the susceptibility of *F. alocis* to neutrophil phagocytosis, internalization of carboxyfluorescein succinimidyl ester (CFSE)-labelled non-opsonized or serum opsonized *F. alocis* (Fig 1A). Phagocytosis of heat-killed *Staphylococcus aureus* served as a control. After 30 min of bacterial challenge and at a multiplicity of infection (MOI) of 10, no statistical difference was observed in the phagocytosis of non-opsonized or opsonized *S. aureus* (Fig. 1B). Human neutrophils showed a similar infection index for both non-opsonized or 10% serum opsonized *F. alocis* (Fig. 1B).

To confirm the imaging flow cytometry observations and distinguish between *F. alocis* associated/attached vs fully engulfed, the neutrophil plasma membrane was stained with wheat germ agglutinin (WGA), and bacteria interactions were visualized by confocal microscopy. Confocal microscopy images showed that when neutrophils were challenged with non-opsonized *F. alocis*, 45% of the cells had internalized bacteria and 54% had only surface-bound/attached bacteria (Fig. 1D). When neutrophils were challenged with opsonized *F. alocis*, 52% of the cells had internalized bacteria, and 45% had only attached bacteria (Fig. 1E). These results indicate that human neutrophils showed similar phagocytic efficiency towards non-opsonized or serum opsonized *F. alocis*.

Survival of *F. alocis* inside human neutrophils

Since human neutrophils effectively phagocytosed *F. alocis*, independent of serum opsonization, we wanted to determine the neutrophil killing efficacy against the organism. Plate counting methodology for *F. alocis* results in a very low percent recovery; therefore, to overcome this limitation, BacLight staining was used to determine bacterial viability (Johnson & Criss, 2013a). Neutrophils were exposed to *F. alocis*, and following synchronized phagocytosis, bacteria viability was determined after 0.5-2-4 h post challenge (Fig. 2A). Our results show that after 30 min of bacterial challenge, 65% of opsonized *F. alocis*, which was attached to and internalized by neutrophils, remained viable; a phenotype that increased to 82% after 2 h, and showed a non significant decrease to 67% after 4 h (Fig. 2B). Currently, there are no antibodies available against *F. alocis*, which would allow to determine extracellular from intracellular bacteria, with more precision. To overcome this limitation, polymerized F-actin was fluorescently labelled, with AlexaFluor 670-Phalloidin, and used in combination with the BacLight staining to determine the percent viability of intracellular *F. alocis*. At 4 h post bacterial challenge the percent of intracellular viable *F. alocis* was $57 \pm 3.8\%$, n=2. Similar percentages of internalized bacterial survival at each

time point were observed when neutrophils were challenged with non-opsonized *F. alocis* (data not shown). The viability of *F. alocis* in the absence of neutrophils, but exposed to the same experimental conditions, was tested using BacLight staining. *F. alocis* viability was similar at 2 h in the presence (82%) or absence of neutrophils (83.5%); however, at 4 h the bacteria viability exposed to neutrophils was reduced (67%), compared to bacteria alone (74%).

To further characterize the subcellular localization of opsonized *F. alocis* within human neutrophils; transmission electron micrograph analyses was performed at 2-4-6-20 h post challenge. At all time points tested, *F. alocis* resided in spacious or semispacious phagosomes (Fig. 2C). At each time point we observed some spacious phagosomes with debris inside, that could be the result of bacterial degradation and/or released of granule content into the organelle. Other phagosomes had neutrophil granules localized in close proximity and contained electron-lucent *F. alocis*, suggestive of a non viable bacterium (Fig. 2C). In contrast, the majority of phagosomes contained electron-dense *F. alocis*, indicative of a viable bacterium, and seemed devoid of neutrophil granules (Fig. 2C). Collectively, these results indicate that *F. alocis* resides inside a phagosomal structure, and that 65% of the intracellular bacterium remained viable 4 h post challenge within phagosomes that showed minimal to no granule recruitment.

***F. alocis* challenge induces minimal neutrophil respiratory burst response**

One of the early and efficient antimicrobial responses that neutrophils mount upon engulfing a bacterium is the robust generation of reactive oxygen species (ROS) through the assembly and activation of the NADPH oxidase multi-subunit enzyme. However, a common bacterial strategy to survive the oxygen-dependent antimicrobial attack from the neutrophils is the inhibition, evasion or neutralization of the NADPH oxidase (Allen, 2003, 2006; Criss & Seifert, 2008). To begin to define the respiratory burst response of human neutrophils when challenged with *F. alocis*, intracellular ROS production was analyzed by flow cytometry. As shown in Fig. 3A, *F. alocis* challenge, in the absence or in the presence of increasing serum concentrations up to 10%, resulted in minimal intracellular ROS production at either 10 or 30 min incubation. Furthermore, the lower passage *F. alocis* D-62D strain, both with and without serum opsonization, showed minimal ROS production similar to the ATCC 35896 type strain (Fig. 3B). As expected, challenge with heat-killed serum opsonized *S. aureus*, used as a positive control, induced a robust intracellular ROS production both after 10 and 30 min incubation (Fig. 3A-B). To further characterize the kinetics of intracellular ROS production by neutrophils when challenged with *F. alocis*, we monitored the respiratory burst response for a total period of 170 min following synchronized phagocytosis (DeLeo, Allen, Apicella, & Nauseef, 1999). Similar to the response observed by flow cytometry (Fig. 3A), both non-opsonized or serum opsonized *F. alocis* elicited minimal ROS production which until 40 min post challenge was similar to basal levels, and then showed a minimal increase between 60 min-80 min followed by a decline back to basal levels by 100 min (Fig. 3C). As expected, the positive control PMA elicited a robust and quick response that peaked by 30 min. By the 60-80 min period of *F. alocis* peak activity, the response elicited by PMA stimulated cells, albeit already at the decline phase, was still three fold higher than the response induced by *F. alocis* (Fig. 3C).

Next, a NBT assay was used to confirm and determine the localization of the minimal intracellular ROS observed in neutrophils challenged with *F. alocis*. Unchallenged neutrophils showed minimal blue formazan deposits (corresponding to basal detection of superoxide levels), in clear contrast to distinct blue deposits of superoxide anions surrounding or inside the majority of the phagosomes containing the controls of opsonized *S. aureus* or opsonized zymosan (Fig. 3D, white arrows). However, minimal blue deposits were observed in neutrophils challenged with *F. alocis* (Fig. 3D, dashed white arrow). A combination of confocal microscopy, using CFSE-labeled 10% opsonized *F. alocis*, and NBT staining was used to confirm that superoxide anions were not forming on *F. alocis* containing phagosomes (Fig. 3D, dashed white arrows).

Depending on the stimuli that neutrophils encounter, the NADPH oxidase complex can also assemble at the neutrophil plasma membrane, resulting in extracellular release of superoxide anions (Nauseef, 2007). Due to the minimal intracellular ROS generated upon *F. alocis* challenge, next we wanted to determine if the organism could stimulate superoxide release. *F. alocis* challenge at any of the three time points tested resulted in minimal superoxide release (Fig. 3E). As expected, in the control condition, stimulation of neutrophils with fMLF induced superoxide release after 5 min of stimulation (Fig. 3E).

To determine if bacterial viability played a role in the modulation of the ROS response, we first wanted to examine the infection index towards live and heat-killed (HK) *F. alocis*. Neutrophils were challenged with either live or HK, CFSE-labelled-*F. alocis*, and internalized bacteria determined by imaging flow cytometry (Fig. 4A). The association of neutrophils with either live or HK *F. alocis* was quantified as an infection index as described in materials and methods. The results showed similar levels of phagocytosis of either live or HK *F. alocis* by human neutrophils (Fig. 4B). To confirm the imaging flow cytometry results and further characterize if live and HK *F. alocis* share a similar pattern of surface bound bacteria only (attached) vs internalized, confocal microscopy was used (Fig. 4C). Quantification of results from 100 neutrophils that were challenged with HK *F. alocis*, from three independent donors, showed that 60% of the cells had internalized bacteria, with a significantly lower percent that was attached only (Fig. 4D). The assays comparing live (non-opsonized or opsonized) vs HK *F. alocis*, in Figures 1 D-E and Fig 4D, were performed on the same day using the same donor's neutrophils, from three independent donors. In supplemental Fig 1A, we plotted the data from Fig 1D-E and Fig 4D, and performed a one-way ANOVA statistical analysis of the different experimental groups. The results showed no statistical difference between the percent of neutrophils with internalized live or HK-*F. alocis* (Supplemental Fig 1A). The percent of attached HK bacteria was significantly lower compared to non-opsonized but not to opsonized *F. alocis* (Supplemental Fig. 1A). To make a further comparison of the bacterial number per cell between live and HK bacterial conditions, the number of neutrophils with 1-2-3-4-5- or 6+ associated and internalized bacteria was determined. No statistical difference was observed between the percent of infected neutrophils with 1 to 5 bacteria for all three bacterial conditions (Supplemental Fig. 1B). However, a significantly higher percent of neutrophils (20%) had 6+ HK bacteria compared to cells with non-opsonized (5%) or opsonized (5%) *F. alocis* (Supplemental Fig. 1B). No significant difference was observed in the percent of infected neutrophils with non-opsonized or opsonized *F. alocis* as well as the total bacterial number per cell (Supplemental

Fig. 1B). The results indicate a similar infection index between live and HK-*F. alocis*; with a higher total bacterial number per infected neutrophil in the HK vs. live bacterial conditions.

To determine if the minimal intracellular ROS production induced by *F. alocis* was actively controlled by the organism, neutrophils were challenged with opsonized (Op)-*F. alocis*, or Op-heat-killed (Op-HK) *F. alocis*. These data show that Op-HK *F. alocis* induced a robust respiratory burst response which was significantly higher than the response induced by the live organism (Fig. 5A). To further examine if the significant difference in the induction of intracellular ROS between live and HK-*F. alocis* was consistent through time, the kinetics of intracellular ROS production was monitored following synchronized phagocytosis (Fig. 5B). Similar to the flow cytometry observations (Fig. 5A), the rate of ROS generated by HK *F. alocis* at its peak period between 40-60 min was more than two fold higher than the maximal response elicited by the live organism (Fig. 5B). In addition, there was a delay in the neutrophils' ability to produce ROS to live *F. alocis* compared to the HK *F. alocis*. The decline in the rate of intracellular ROS by both live and HK *F. alocis* reached basal levels by 100-120 min (Fig. 5B). Overall, these results indicate that live *F. alocis* is able to manipulate and prevent the respiratory burst response.

To further characterize if the minimal intracellular ROS production induced by *F. alocis* was dependent on the amount of bacteria exposed to neutrophils, kinetic assays were performed with increasing doses of both non-opsonized and opsonized *F. alocis*. Our results show a similar dose dependent increase in ROS production by non-opsonized and opsonized *F. alocis* (Supplemental Fig 2A-B). Moreover, similar to the results obtained with MOI 10, the HK *F. alocis* induced significantly higher intracellular ROS production compared to live organisms at both MOI of 50 and 100 (Supplemental Fig 2C-D). These results indicate that *F. alocis*, with or without opsonization, elicits a minimal ROS production at an MOI of 10. Increasing the dose of *F. alocis* results in a higher production of reactive oxygen species, but that response remains significantly lower compared to HK organisms.

As no difference was observed between non-opsonized and serum opsonized *F. alocis* in the percent of infection as well as the total bacterial number per cell, all further experiments were conducted with opsonized bacteria at an MOI of 10, unless indicated otherwise. The observations that only the viable bacteria were capable of preventing ROS generation, led to the hypothesis that secreted products of *F. alocis* might be responsible for this effect. To examine if the inability of *F. alocis* to generate ROS was mediated by bacterial secreted products, culture supernatants were collected. The ability to generate ROS was tested in neutrophils pre-treated with *F. alocis* supernatant followed by *F. alocis* or *S. aureus* challenge, and compared to each bacterial stimulus alone. The data showed that pretreatment with the *F. alocis* culture supernatant resulted in a significantly higher respiratory burst response induced by *S. aureus* than the response elicited by *S. aureus* alone (Fig. 5C). Minimal ROS generation was observed by neutrophils exposed to the culture supernatant alone (Fig. 5C). This result suggests that *F. alocis* secreted products are not involved in the low ROS levels generated in *F. alocis* challenged neutrophils. During an inflammatory setting *in vivo*, quiescent neutrophils can change into a pre-activated phenotype or primed cell due to exposure to inflammatory cytokines such as TNF- α , interleukin (IL)-1 β and IL-17 (Kruger, Saffarzadeh, Weber, Rieber, Radsak, von Bernuth, ... Hartl, 2015; Miralda,

Uriarte, & McLeish, 2017). Primed neutrophils have a more robust response when encountering a second stimulus, but a priming agent, such as TNF- α , by itself does not result in generation of oxidants (Condliffe, Kitchen, & Chilvers, 1998). These results would indicate that *F. alocis* secreted products, exhibit a priming response for neutrophils as opposed to an inhibitory one.

Since secreted bacterial products had no impact on the respiratory burst response induced by *F. alocis* challenge, we sought to test if the presence of live bacteria was necessary to inhibit the respiratory burst in response to known agonists of NADPH oxidase activity. To test this hypothesis, neutrophils were challenged with *F. alocis* (MOI 10), or *S. aureus* (MOI 10) for 15 min followed by 30 min infection by *S. aureus* (MOI 10). Also, cells were challenged by a co-infection condition to determine if the MOI of total bacteria made a difference in the cells' response. In this "F.a + S.a" condition, *F. alocis* (MOI 5) and *S. aureus* (MOI 5) were added together for a total MOI of 10. Surprisingly, neither inhibition nor priming of the respiratory burst response was noticed when neutrophils were pretreated with *F. alocis* followed by *S. aureus* as a second stimuli (Fig. 5D). The slight, although significant, increase in ROS generation observed with *F. alocis* pre-treatment followed by *S. aureus* can be attributed to the greater total amount of bacteria per neutrophil (MOI 20). The co-infection condition (F.a + S.a) gave values that were not significantly different from the *S. aureus* alone condition, reflective of the effect induced by the response from each bacterium (Fig. 5D). To further characterize the effect of *F. alocis* on the respiratory burst response in the *S. aureus* co-infected condition; NBT staining combined with confocal microscopy was performed (Supplemental Fig. 3A). The data showed positive accumulation of superoxide, as indicated by formation of black deposits in 78% of opsonized-*S. aureus* phagosomes and only on 29 % of *F. alocis* phagosomes. A similar level of NBT positive *F. alocis* phagosomes ($27 \pm 3.7\%$, n=4) was observed in neutrophils infected only with *F. alocis* (Supplemental Fig. 3B). Recently our laboratory reported that another emerging periodontal pathogen, *Peptoanaerobacter stomatis*, is able to elicit a robust intracellular ROS production by human neutrophils (Jimenez Flores, Tian, Sizova, Epstein, Lamont, & Uriarte, 2017). Hence, we next examined if *F. alocis* could modulate the ROS production induced by *P. stomatis* (Fig. 5E). Similar to the situation with *S. aureus*; the results obtained with the co-infection condition (F.a + P.s) would suggest an additive effect of the response elicited by each oral species (Fig. 5E). Overall our results indicate that *F. alocis* fails to mount an oxidative response, but does not inhibit the neutrophil respiratory burst to other particulate stimuli. In summary, our data show that the failure to activate the respiratory burst is specific to *F. alocis*-containing phagosomes and is not a global effect within the infected neutrophil.

Partial recruitment of both specific and azurophil granules to *F. alocis*-containing phagosomes

Upon internalization, maturation of the phagosome is dependent on specific and azurophil granule fusion (Nordenfelt & Tapper, 2011). Specific granules contains 60% of the membrane bound components of the NADPH oxidase complex (gp91 $phox$ and p22 $phox$) which is responsible for ROS generation (Borregaard, Sørensen, & Theilgaard-Mönch, 2007). Other neutrophil granules such as the secretory vesicles and gelatinase granules also contain these membrane oxidase components, but these granules are largely exocytosed and

are not typically targeted to the phagosome (Borregaard, Sørensen, & Theilgaard-Mönch, 2007; Sengelov, Kjeldsen, & Borregaard, 1993). To determine if *F. alocis*' failure to induce a robust ROS generation was due to an inhibition of specific granule fusion to the bacteria-containing phagosome, we examined the recruitment of lactoferrin, a marker for this granule subtype, to the bacteria-containing phagosome (Allen, Beecher, Lynch, Rohner, & Wittine, 2005). Neutrophils were left untreated, or were challenged with AlexaFluor488-labeled *S. aureus*, or CFSE-labeled Op-*F. alocis*, or CFSE-labeled Op-HK *F. alocis*, and analyzed by confocal microscopy. The bacteria-containing phagosome was considered positive for the granule marker if 50% of the phagosome was enriched for it (Johnson & Criss, 2013b). For each of three different donors, 100 infected and intact cells were quantified for lactoferrin positive and negative phagosomes. The results showed that 66-70% of both *S. aureus* and heat-killed *F. alocis*-containing phagosomes were lactoferrin positive, whereas a significantly lower percent of *F. alocis*-containing phagosomes (36.6%) was able to recruit the granule marker (Fig. 6A and B). In addition, the confocal images show similar levels of uptake of live and dead *F. alocis* by the neutrophils (Fig. 6A). To determine if *F. alocis* was preventing or delaying specific granule recruitment to the phagosome, the percent of lactoferrin enrichment to the bacterial phagosome was quantified after 2 h of infection using the same criteria performed for 30 min time point. The percent of lactoferrin enrichment on *F. alocis* phagosomes significantly increased from $36.6 \pm 1.7\%$ after 30 min to $67.2 \pm 2.9\%$ ($p < 0.0031$, $n=3$). These results indicate that live *F. alocis* delays specific granule fusion to its phagosomes within human neutrophils.

In neutrophils, mobilization and fusion of azurophil granules to bacteria-containing phagosomes marks a key step in phagosome maturation and efficient microbial killing (Nauseef, 2007; Nordenfelt & Tapper, 2011). We next examined if *F. alocis* could also control azurophil granule recruitment to its phagosomes. The same criteria used for lactoferrin recruitment were applied to the azurophil granule marker, neutrophil elastase. The data showed similar percent recruitment of elastase to phagosomes containing *S. aureus* (62%) and heat-killed *F. alocis* (70%), with a significantly lower percent of *F. alocis* phagosomes (41%) enriched for the azurophil marker (Fig 7 A and B). Since we observed that *F. alocis* was delaying specific granule recruitment to its phagosome, we next wanted to determine if a similar response would occur with the recruitment of azurophil granules to the bacteria containing phagosome. In contrast to the increase in specific granule recruitment to *F. alocis* phagosomes after 2 h of cell challenge, the percent of elastase enrichment on *F. alocis* phagosomes remain similar between 30 min ($41 \pm 7.3\%$, $n=3$) and 2 h ($46 \pm 2.1\%$, $n=3$). Overall, our results indicate that *F. alocis* delays specific granule fusion to its phagosome and prevents azurophil granule recruitment, resulting in lower ROS generation as well as less exposure to the highly microbicidal content present inside those two granule subtypes.

Except for secretory vesicles, live and HK *F. alocis*, promote a similar granule exocytosis response

Our results in Figure 6A show that live *F. alocis* activates the neutrophil by moving the specific granules to the periphery of the cell instead of towards the bacteria-containing phagosome. Upon neutrophil activation, the different granule subtypes can be mobilized

towards the bacteria-phagosome and/or to the cell plasma membrane and their content released to the extracellular space through a process of stimulated exocytosis. The granule exocytosis process is sequentially controlled with the earliest formed granules during granulopoiesis being the last to be released (Borregaard, Sørensen, & Theilgaard-Mönch, 2007; Sengelov, Kjeldsen, & Borregaard, 1993). The earlier formed granules include the azurophil and specific granules which contain the most microbicidal and cytotoxic components (Borregaard, Sørensen, & Theilgaard-Mönch, 2007). The later formed granules—gelatinase and secretory vesicles—are more involved in the process of chemotaxis, adhesion to the endothelium and extravasation through blood vessels to the site of infection (Borregaard, Kjeldsen, Sengelov, Diamond, Springer, Anderson, ... Bainton, 1994; Borregaard, Sørensen, & Theilgaard-Mönch, 2007).

We recently described that non-opsonized *F. alocis*, through TLR2 activation, promotes exocytosis of secretory vesicles and specific granules but not azurophil granules in human neutrophils; as well as enhanced random and directed migration (Armstrong, Miralda, Neff, Tian, Vashishta, Perez, ... Uriarte, 2016). We next examined if opsonized HK *F. alocis* would induce a similar or distinct granule exocytosis pattern compared to the live organism. Neutrophils were unstimulated, or stimulated with the positive control, fMLF, or challenged with opsonized *F. alocis*, or HK opsonized *F. alocis*. Exocytosis of secretory vesicles and specific granules was determined by flow cytometry and identified by CD35 and CD66b plasma membrane expression respectively. As expected, fMLF triggered a robust and quick increase of the plasma membrane expression of both CD35 and CD66 (Fig. 8A and B). Similar to the response we described with non-opsonized *F. alocis* (Armstrong, Miralda, Neff, Tian, Vashishta, Perez, ... Uriarte, 2016); serum opsonized *F. alocis* significantly induced secretory vesicles and specific granule exocytosis (Fig. 8A and B). In contrast, HK *F. alocis* did not induce secretory vesicle exocytosis (Fig. 8A). However, both the live and heat-killed opsonized *F. alocis* induced a significant increase of CD66b membrane expression (Fig. 8B).

The gelatinase granule's exocytosis is critical to the extravasation of neutrophils from the bloodstream to the tissue (Borregaard, Sørensen, & Theilgaard-Mönch, 2007). In addition, the matrix content of this granule subtype is enriched with matrix metalloproteinases (MMPs) which are detected in the supernatant collected from neutrophils stimulated with periodontal pathogens. Furthermore, MMPs are detected in high numbers in the gingival tissue from periodontitis patients and are considered important contributors to the gingival tissue damage observed in these patients (Ding, Haapasalo, Kerosuo, Lounatmaa, Kotiranta, & Sorsa, 1997; Smith, Muñoz, Collados, & Oyarzún, 2004). Stimulation of human neutrophils with either live or HK opsonized *F. alocis* resulted in similar release of MMP-9, comparable to the response induced by fMLF (Fig. 8C). Although neutrophil challenge with the HK *F. alocis* showed a slight increase in gelatinase granule exocytosis compared to live *F. alocis*, those differences did not achieve statistical significance (Fig. 8C). To further characterize if the induction of gelatinase granule exocytosis by live or HK *F. alocis* resulted in the release of active matrix metalloproteinases, gelatin zymography was employed. Our results show a similar band intensity at 92 kDa, which corresponds to the molecular weight size of MMP-9, for both live and HK *F. alocis*-challenged neutrophils, as well as for the positive control, fMLF (Fig. 8D and E). In addition, the supernatants collected showed a

higher molecular weight band around 130 kDa potentially corresponding to an MMP-9 dimer with neutrophil gelatinase associated lipocalin (NGAL) as previously reported (Mair, Jhamb, Visser, Aguirre, & Kramer, 2016).

The neutrophil granule that can cause the most tissue damage if its content is released outside the cell is the azurophil granule (Borregaard, Sørensen, & Theilgaard-Mönch, 2007). Perhaps due to the highly toxic cargo, these granules are difficult to induce to undergo exocytosis, and they are primarily targeted to the phagosome (Amulic, Cazalet, Hayes, Metzler, & Zychlinsky, 2012; Sengelov, Kjeldsen, & Borregaard, 1993). These granules contain myeloperoxidase which is involved in the formation of hypochlorous acid (HOCl) within the phagosome; therefore, they are critical to the oxidative burst response and to phagosome maturation (Nauseef, 2007). Our laboratory recently reported that challenge of human neutrophils with non-opsonized *F. alocis* did not induce azurophil granule exocytosis (Armstrong, Miralda, Neff, Tian, Vashishta, Perez, ... Uriarte, 2016). Our confocal results from Fig. 7 show significantly lower recruitment of azurophil granules to *F. alocis* phagosomes compared to cells challenged with the HK organisms. To determine if serum opsonized-*F. alocis* would promote azurophil granule exocytosis; the increase of the granule marker, CD63, on the neutrophil plasma membrane was determined by flow cytometry. The results show that neither live nor HK opsonized *F. alocis* induced a significant increase of the expression of the granule marker CD63 compared to basal levels (Fig. 8F). Overall these results indicate that bacterial viability plays a role in the stimulation of secretory vesicle exocytosis. However, both live and HK opsonized *F. alocis* stimulated gelatinase granules and specific granule exocytosis; and failed to induce azurophil granule exocytosis.

Discussion

Neutrophils are recruited to the periodontal pocket and subsequently the crevicular fluid to form a “wall” to protect against microbial intrusion of the deeper tissue (Darveau, 2009; Ryder, 2010). The crevicular fluid becomes rich in neutrophils actively secreting their granule contents, generating reactive oxygen species, and even releasing their DNA in the process of neutrophil extracellular trap (NET) formation, all of which contribute to tissue damage and non-resolving inflammation (Hirschfeld, J., Dommisch, H., Skora, P., Horvath, G., Latz, E., Hoerauf, A., ... Bekeredjian-Ding, I., 2015; Vitkov, Klappacher, Hannig, & Krautgartner, 2010; White, Chicca, Cooper, Milward, & Chapple, 2016). The etiology of periodontal disease can be viewed as a shift from an indigenous symbiotic oral community into a more diverse, dysbiotic one which can modulate the immune system to sustain chronic inflammation and disease progression (Hajishengallis & Lamont, 2016). How periodontal pathogens can evade and/or manipulate the innate immune system is of relevance to the development of therapeutic strategies to combat periodontal disease. The emerging oral pathogen, *F. alocis*, is found in high numbers in periodontal disease sites compared to healthy sites, an indication that it can manipulate the innate immune response (Abusleme, Dupuy, Dutzan, Silva, Burleson, Strausbaugh, ... Diaz, 2013; Dahlen & Leonhardt, 2006; Kumar, Griffen, Moeschberger, & Leys, 2005). The aim of the present study was to determine if *F. alocis* could withstand neutrophils' potent antimicrobial mechanisms. Our results show that human neutrophils can engulf with the same efficiency both non-opsonized as well as serum opsonized *F. alocis*. Moreover, more than 60% of *F. alocis*, attached to and

internalized by human neutrophils, remains viable four hours post infection. Recently, a protein present on the surface of *F. alocis*, FACIN, was characterized as an inhibitor of complement activation allowing the organism to reduce opsonization and to withstand complement attack (Jusko, Miedziak, Ermert, Magda, King, Bielecka, ... Blom, 2016). Therefore, it appears that *F. alocis* has evolved several strategies to resist neutrophil action. First, *F. alocis* can prevent complement activation and reduce its opsonization; however human neutrophils can efficiently engulf it in the absence or in low percent of complement deposition, hence the oral pathogen deploys the next evasion strategy inside the professional phagocyte by preventing ROS generation and granule recruitment to its phagosome.

As professional phagocytes, neutrophils rapidly and efficiently engulf microorganisms and initiate their antimicrobial attack by generation of ROS through activation of the NADPH oxidase complex and by mobilization of their cytosolic granules towards the forming phagosome (Nauseef, 2007). The interaction between neutrophils and the keystone oral pathogen, *P. gingivalis*, results in significant intracellular ROS production (Jayaprakash, Demirel, Khalaf, & Bengtsson, 2015). In addition, *P. gingivalis* can induce similar levels of intracellular ROS from neutrophils isolated from healthy donors or from patients with localized aggressive periodontitis (Damgaard, Kantarci, Holmstrup, Hasturk, Nielsen, & Van Dyke, 2017). Another study compared the activation of the respiratory burst response when neutrophils were challenged with different periodontal pathogens; *Fusobacterium nucleatum* showed the highest intracellular and extracellular generation of ROS compared to *P. gingivalis* and *Aggregatibacter actinomycetemcomitans* (Katsuragi, Ohtake, Kurasawa, & Saito, 2003). Recently our laboratory showed that *P. stomatis*, an emerging oral pathogen, induces robust intracellular ROS production in human neutrophils, albeit the oxygen dependent response was not responsible for the antimicrobial activity (Jimenez Flores, Tian, Sizova, Epstein, Lamont, & Uriarte, 2017). In contrast, our results show that phagocytosis of *F. alocis* causes minimal intracellular and extracellular generation of reactive oxygen species; a phenotype, which to the best of our knowledge, is unique to *F. alocis* compared to other periodontal pathogens.

Neutrophils have the capacity to efficiently and quickly kill bacteria within 30 to 60 min post-infection (Nauseef, 2007). However, several pathogenic microorganisms deploy different strategies to hijack neutrophil oxygen dependent and/or independent antimicrobial mechanisms by targeting the NADPH oxidase complex to prevent ROS generation or inhibit granule recruitment to the bacterial phagosome (Allen & McCaffrey, 2007; Johnson & Criss, 2011; Kinkad & Allen, 2016). The survival of *F. alocis* inside neutrophils may be partially explained by the delay of specific granules and prevention of azurophil granule fusion to the phagosome. In addition, *F. alocis* does not suppress the respiratory burst induced by heat-killed *S. aureus* or the live *P. stomatis*; suggesting that inhibition of ROS production is not a global mechanism, but rather a local phagosomal mechanism to promote survival. Similar manipulation of the respiratory burst response has been described for *Anaplasma phagocytophilum* which induces minimal ROS production in human neutrophils without inhibiting the response elicited by *E. coli* in co-infection studies (Ijdo & Mueller, 2004). *Coxiella burnetii* also mounts a minimal respiratory burst response upon phagocytosis by human neutrophils but does not prevent the fMLF or PMA-stimulated response (Siemsen, Kirpotina, Jutila, & Quinn, 2009). Other bacteria such as *Francisella tularensis* (McCaffrey

& Allen, 2006) and *Neisseria gonorrhoeae* lacking opacity-associate (Opa) proteins (Smirnov, Daily, & Criss, 2014), prevent assembly of the NADPH oxidase complex at the bacteria-containing phagosome, which allows *F. tularensis* to escape and proliferate within the neutrophil cytosol; and *N. gonorrhoeae* to survive inside neutrophils and to inhibit fMLF and serum-opsonized *S. aureus*-induced respiratory burst response, but not the response elicited by PMA stimulation (Criss & Seifert, 2008). Our results indicate that *F. alocis* minimal ROS production could be related to the low percent of lactoferrin-positive bacteria-containing phagosomes (Borregaard, Sørensen, & Theilgaard-Mönch, 2007). In addition, delaying specific granule fusion with *F. alocis* phagosomes will also diminish the release of antimicrobial peptides, such as lysozyme and lactoferrin, inside the phagosome, thus promoting bacterial survival. It also possible that recruitment of the NADPH oxidase cytosolic components or their phosphorylation status might be manipulated by *F. alocis*, and studies to address this topic are ongoing in our laboratory.

Preventing fusion of neutrophil granules to bacterial phagosomes is an important strategy to delay phagosome maturation and allow bacterial survival (Nordenfelt & Tapper, 2011). *N. gonorrhoeae* can delay azurophil granule fusion which is a key step to achieve phagosome maturation (Johnson & Criss, 2011; Johnson & Criss, 2013b). In our study, *F. alocis* prevented azurophil granule fusion to the phagosome to avoid phagosome maturation and survive inside the neutrophils. The mechanism(s) which are deployed by *F. alocis* to prevent granule trafficking and/or fusion to the phagosome are currently under investigation in our laboratory.

We recently found that non-opsonized *F. alocis* triggers secretory vesicles and specific granule exocytosis in a TLR2-depended manner, but does not induce azurophil granule release (Armstrong, Miralda, Neff, Tian, Vashishta, Perez, ... Uriarte, 2016). In the present study, we extended these findings to show that 10% serum opsonized *F. alocis* induced secretory vesicles, gelatinase and specific granule exocytosis, but not azurophil granules; a process that was not dependent on bacterial viability except in the case of the secretory vesicles. Degranulation has been shown to be a critical step in the priming and activation of neutrophils (Miralda, Uriarte, & McLeish, 2017; Uriarte, Rane, Luerman, Barati, Ward, Nauseef, & McLeish, 2011). Previous studies have shown that *F. alocis* induced the release of pro-inflammatory cytokines such as TNF- α , IL-1 β and IL-6 by epithelial cells which could result in priming and/or activation of human neutrophils (Moffatt, Whitmore, Griffen, Leys, & Lamont, 2011). Our results show that once the neutrophils have encountered *F. alocis*, further degranulation events and activation occur. With the influx of cytokine signals from the epithelium and the exogenous activators from *F. alocis*, the neutrophil may become extremely activated and localized to the infected periodontal pocket.

The exocytosis of neutrophil matrix metalloproteases, such as MMP-9, and other serine proteases contributes significantly to the tissue damage associated with periodontal disease. Several periodontal pathogens induce release of MMP-9 and elastase (Ding, Haapasalo, Kerosuo, Lounatmaa, Kotiranta, & Sorsa, 1997). Recently, we reported that *P. stomatis* induced exocytosis of the four neutrophil granules in a time and dose dependent manner (Jimenez Flores, Tian, Sizova, Epstein, Lamont, & Uriarte, 2017). In the present study we show that both live and heat-killed *F. alocis* are able to induce release of active MMP-9. In

addition to augmenting the pool of proteases in the gingival tissue, the exocytosis of neutrophil granules increases the NADPH oxidase components on the plasma membrane which results in increased superoxide release in the extracellular milieu (Kruger, Saffarzadeh, Weber, Rieber, Radsak, von Bernuth, ... Hartl, 2015). Reactive oxygen species such as superoxide alone are sufficient to damage the periodontal tissues (Scott & Krauss, 2012). Our investigation of *F. alocis* has shown active exocytosis of the three neutrophil granules which contain NADPH oxidase components. Additionally, the secreted products of *F. alocis* were found to prime neutrophils for an enhanced respiratory burst response. Collectively, these results indicate a possible role for *F. alocis* in increasing the inflammation and tissue damage within the periodontal pocket. Other investigations have shown that *F. alocis* is largely localized to the areas of the biofilm in contact with the soft tissue meaning that it is likely to be in direct contact with the neutrophil wall (Schlafer, Riep, Griffen, Petrich, Hübner, Berning, ... Moter, 2010).

The failure of *F. alocis* to mount a robust respiratory response also has implications for longevity of the neutrophils. The generation of ROS within neutrophils eventually results in apoptosis and clearance by resident macrophages (Fox, Leitch, Duffin, Haslett, & Rossi, 2010). This normal sequence of events prevents prolonged inflammation and tissue damage (Fox, Leitch, Duffin, Haslett, & Rossi, 2010). Other bacterial species such as *F. tularensis* and *N. gonorrhoeae* have been shown to inhibit oxidase activity to delay apoptosis and thus contribute to prolonged inflammation (Schwartz, Barker, Kaufman, Fayram, McCracken, & Allen, 2012; Simons, Nauseef, Griffith, & Apicella, 2006; Chen & Seifert, 2011). In fact, induction of neutrophil apoptosis has been proposed as a therapeutic for chronic inflammatory conditions (Hampson, Hazeldine, & Lord, 2013).

Neutrophils are the main innate immune cell that patrols the gingival tissue in both health and in disease. This study provides some novel insights into the outcome of the encounter between neutrophils and *F. alocis*, an organism that is a member of dysbiotic microbial communities. *F. alocis* can manipulate antimicrobial responses, by inducing minimal respiratory burst activation, and preventing specific and azurophil granule recruitment to its phagosome, which will facilitate increased bacterial survival. Subsequently, neutrophil granules involved in neutrophil recruitment, migration (secretory vesicles, gelatinase and specific) are mobilized and these can contribute to tissue damage. Despite granule exocytosis contributing to neutrophil activation and priming, *F. alocis* is recalcitrant to neutrophil killing.

Experimental Procedures

Human neutrophil isolation

Blood was drawn from healthy donors and neutrophils were purified using plasma-Percoll gradients as previously described (Uriarte, Rane, Luerman, Barati, Ward, Nauseef, & McLeish, 2011), and in accordance with the guidelines approved by the Institutional Review Board of the University of Louisville. The purity of the isolated cell fraction was determined by microscopic evaluation and showed that 95% of the cells were neutrophils. Cell viability was confirmed by trypan blue exclusion indicated that 97% of cells were viable.

Bacterial strains and growth conditions

F. alocis ATCC 38596 and low passage clinical isolate D-62D were cultured in brain heart infusion (BHI) broth with yeast extract supplemented with L-cysteine (0.1%) and arginine (0.05%) for 14 days anaerobically at 37°C as previously described (Aruni, Roy, & Fletcher, 2011; Moffatt, Whitmore, Griffen, Leys, & Lamont, 2011). Heat killed *F. alocis* was generated by incubation at 90 °C for 60 min. Non-viability was confirmed by incubation in culture media at same conditions used for the live organism. Serum opsonization was performed by incubation of *F. alocis* at 37°C for 20 min in different percentages, 2-5-10%, of normal human serum (Complement Technology, Inc.) For fluorescence immunostaining assays, *F. alocis* was labeled with carboxyfluorescein succinimidyl ester (CFSE; 40 ng/μl) for 30 min at room temperature in the dark, and washed 3 times with PBS prior to use.

Peptoanaerobacter stomatis strain CM2 was cultured anaerobically at 37°C in Tryptic Soy Broth supplemented with 20 g/L yeast extract, 1% hemin and 1% reducing agent (37.5 g/L NH₄Cl, 25 g/L MgCl₂ × 6H₂O, 5 g/L CaCl₂ × 2H₂O, 50 g/L L-cysteine HCl, 5 g/L FeCl₂ × 4H₂O) as previously described (Jimenez Flores, Tian, Sizova, Epstein, Lamont, & Uriarte, 2017).

Phagocytosis of *F. alocis* by human neutrophils

a) Imaging flow cytometry—Human neutrophils (4×10^6 cells/ml) were challenged with either human serum opsonized or with non-opsonized CFSE-labeled *F. alocis*, or CFSE-labeled HK-*F. alocis* at a multiplicity of infection (MOI) of 10. As a control, neutrophils were challenged with either human serum opsonized, or non-opsonized, Alexa Fluor 488 heat-killed *Staphylococcus aureus* (Invitrogen; MOI 10). Cells were incubated in a shaking water bath at 37°C for 30 min, pelleted at 6000g for 30 seconds followed by rinsing with 0.05% sodium azide and fixing with 1% paraformaldehyde. Neutrophils were incubated with labeled bacteria (Alexa Fluor 488-*S. aureus* or CFSE-labelled -*F. alocis*) at 4°C to detect the surface bound/attached labeled bacteria only. An infection index was calculated to normalize the Alexa Fluor 488/CFSE positive cells distinguishing between internalized vs surface bound/attached bacteria. The infection index was calculated by: (the percent of Alexa Fluor 488/CFSE positive neutrophils x mean fluorescence intensity at 37°C – the percent of Alexa Fluor 488/CFSE positive neutrophils x mean fluorescence intensity at 4°C); as previously reported (Schwartz, Barker, Kaufman, Fayram, McCracken, & Allen, 2012; Kinkad, Fayram, & Allen, 2017). For some experiments, the neutrophil plasma membrane was stained for 10 min with wheat germ agglutinin (WGA, Invitrogen). Images were obtained, quantified and visualized using an Amnis ImageStream^X (Millipore). Labeled bacterial internalization was detected using excitation with a 488 nm solid-state laser, and WGA staining was detected using excitation with a 642 nm solid-state laser. A total of 1000 neutrophil events were collected per condition and sorted into bacteria positive or negative bins based on CFSE/AlexaFluor488 intensity. The data were analyzed using the IDEAS Application v6.0 software (Amnis-Millipore). For each experiment, the internalization wizard was used to design a mask to count the CFSE or Alexa Fluor 488 positive cells and to ignore positive signals from membrane-associated or extracellular bacteria. The Erode mask (Erode 4) was created based on the Idea software user manual to remove the selected number of pixels from all edges of the starting mask.

b) Confocal microscopy—Human neutrophils (2×10^6 cells/ml) were stimulated with human serum opsonized or with non-opsonized CFSE-labeled *F. alocis*, or CFSE-labeled HK-*F. alocis* at MOI of 10 and incubated in a shaking water bath at 37°C for 30 min. After incubation, the samples were rinsed once with RPMI media, transferred to glass cover slips and centrifuged at 600 x g for 8 min at 4°C. Cells were fixed with 10% formalin, and the cell plasma membrane stained with WGA (Invitrogen). Images were acquired by Fluoview FV1000 confocal microscope and analyzed by FV-10ASW software (Olympus). To quantify the percent of infection, 100 neutrophils were examined per condition and the percent of cells with bacteria internalized, or attached to the plasma membrane, or both was calculated.

Bacterial viability assay

Human neutrophils (2×10^6 cells/ml) were seeded to glass cover slips coated with pooled human serum (Sigma S7023) and incubated at 37°C for 30 min to allow adherence to the coverslips; followed by *F. alocis* challenge for 0.5-2-4 hours. Bacteria viability was determined by using the combination of two DNA dyes, SYTO9 (5 µM) and propidium iodide (30 µM) as previously described (Johnson & Criss, 2013a). The appropriate controls of live and heat killed bacteria alone were used to standardize the DNA dyes concentrations. Bacterial cells with intact membrane stain green by SYTO9 and bacterial cells with compromise membrane will be permeable to propidium iodide and stain red. In some experiments, after the different bacterial challenge time points, cells were stained for 30 min with AlexaFluor 670-Phalloidin (which binds polymerized F-actin) to discriminate between extracellular vs intracellular bacteria; followed by the live/dead staining. Confocal images were acquired within using an Olympus Fluoview FV1000 confocal microscope and analyzed by FV-10ASW software. Quantification was performed by counting the total viable and non-viable bacteria both attached to and internalized from 100 neutrophils in 3 independent experiments.

Transmission electron microscopy

Neutrophils (4×10^6 cells/ml) were challenged with *F. alocis* for 2-4-6-20 hours at 37 °C in 5% CO₂ incubator. At the appropriate time points, the cells were fixed with freshly made 3 % glutaraldehyde in 0.1 M PO₄ buffer pH 7.3; and submitted to the University of Louisville Department of Anatomical Sciences and Neurobiology Microscopy Core Facility for transmission electron microscopy processing and analysis. The samples were then post fixed in 1% OsO₄, dehydrated in ascending grades of ETOH, then exchanged with propylene oxide and embedded in EMbed812 from Electron Microscopy Sciences. Samples were cut on a Reichert-jung ultracut-E ultramicrotome with a diamond knife. Gold sections (90 nm thick) were obtained. Sections were stained with uranyl acetate and Reynolds lead citrate. Images were acquired on a Phillips CM10 TEM at 80 kv.

Respiratory burst response

a) Intracellular respiratory burst response—Neutrophils (4×10^6 cells/ml) were challenged with either non-opsonized or serum opsonized *F. alocis*, or with *S. aureus* at MOI 10, for 10 min or 30 min. For some experimental conditions, the supernatant from *F. alocis* culture was collected, and passed through a low binding 0.22 µm-pore filter to remove

bacterial cells. Neutrophils were pre-treated with the bacterial supernatant for 15 min prior to *S. aureus* challenge. The phagocytosis-stimulated respiratory burst response was measured by 2', 7'-dichlorofluorescein (DCF, 5 μ M) and analyzed by flow cytometry using a BD FACS Calibur™ as previously described (Uriarte et al., 2011). The nitroblue tetrazolium (NBT) assay was also used to visualize intracellular superoxide radical production induced by *F. alocis*. Neutrophils (1×10^6 cell/ml) attached to plasma-coated glass coverslips were unstimulated or stimulated with opsonized *S. aureus* or with opsonized zymosan (Invitrogen), or with opsonized *F. alocis* or opsonized CFSE-labelled *F. alocis*. Phagocytosis was synchronized at $600 \times g$, 14°C for 4 min and cells were incubated at 37°C in 5% CO₂ for 60 min in RPMI containing NBT. After incubation, cells were fixed with methanol and analyzed by light or confocal microscopy. Reduced NBT precipitates were visualized as blue formazan deposits.

A kinetic intracellular reactive oxygen species (ROS) production in neutrophils was measured as previously described (DeLeo, Allen, Apicella, & Nauseef, 1999) with modifications. Neutrophils were resuspended in DPBS with Ca⁺ and Mg⁺ containing 25 μ M DCF (Molecular Probes) to 10^7 cells/ml for 20-25 min at room temperature with gentle agitation. DCF-containing neutrophils (10^6), 8.0 μ g of superoxide dismutase (SOD), and opsonized *F. alocis* or non-opsonized *F. alocis* or heat-killed *F. alocis* (MOI of 10, 50, 100), or 0.2 μ g of phorbol-12-myristate-13-acetate (PMA; Sigma) were combined in wells of a 96-well microtiter plate at 14°C, centrifuged for 4 min at $600 \times g$, and transferred to a microplate fluorometer (Victor™ X3; Perkin Elmer). ROS production was measured continuously at 1-min intervals for up to 180 min at 37°C using excitation and emission wavelengths of 485 and 538 nm, respectively. The rate of neutrophil ROS production over time was determined from the average fluorescence for triplicate wells within each 10-min time period.

b) Extracellular respiratory burst response—Neutrophils (4×10^6 cells/ml) were left unstimulated or were challenged with fMLF (Sigma; 300 nM) for 5 min, or with *F. alocis* for 5, 30, or 60 min at 37°C. After stimulation, the samples were centrifuged for 10 min at $600 \times g$, 4 °C and supernatants were collected. Superoxide anion release was measured spectrophotometrically at 550 nm as the superoxide dismutase-inhibitable reduction of ferricytochrome *c* as previously described (Uriarte, Rane, Luerman, Barati, Ward, Nauseef, & McLeish, 2011).

Immunostaining of specific and azurophil granule recruitment

Specific and azurophil granule fusion to bacteria-containing phagosomes was tested by immunostaining using confocal microscopy as previously described (Jimenez Flores, Tian, Sizova, Epstein, Lamont, & Uriarte, 2017; Johnson & Criss, 2013b). Neutrophils (2×10^6 cells/ml) were unstimulated, or challenged with opsonized CFSE- labeled *F. alocis*, opsonized CFSE-labeled HK-*F. alocis*, or AlexaFluor 488 labeled heat-killed *S. aureus* for 30 and 120 min. After the indicated time point, cells were fixed in 10% formalin, permeabilized in acetone: methanol at -20°C, and blocked in PBS containing 5% bovine serum albumin for 1 h at room temperature. Recruitment of specific granules to bacteria-containing phagosomes was determined with anti-human lactoferrin primary antibody (MP

Biomedicals). Azurophil granule recruitment was determined with anti-neutrophil elastase antibody (AHN-10, Millipore). The secondary antibody utilized was Alexa Fluor 647 (Life Technologies) and DAPI was used to stain the cell nucleus. Confocal images and Z-stacks (1 μ m thickness for each slice) were obtained using a Fluoview FV1000 confocal microscope with a 63x oil objective. To quantify the enrichment of the bacteria-containing phagosomes for a particular granule subtype, 100 neutrophils in each experimental condition were counted and if 50% of the phagosome was surrounded by lactoferrin or elastase, it was considered positive granule marker recruitment, as previously described (Jimenez Flores, Tian, Sizova, Epstein, Lamont, & Uriarte, 2017; Johnson & Criss, 2013b).

Neutrophil granule exocytosis

Neutrophils (4×10^6 cells/ml) were unstimulated (basal), or stimulated with fMLF (300 nM, 5 min) or challenged with *F. alocis* or HK-*F. alocis* at MOI of 10 for 30 min; or pretreated with TNF- α (R&D Systems; 2 ng/mL, 10 min) followed by a fMLF (300 nM, 5 min). Exocytosis of azurophil granules, specific granules, and secretory vesicles was determined by measuring the increase in plasma membrane expression of membrane-associated receptors using antibodies: FITC-conjugated CD63 (Ancell Corporation, clone AHN16.1/46-4-5), FITC-conjugated anti human CD66b (Biolegend, clone G10F5), and PE-conjugated anti-human CD35 (Biolegend, clone E11), respectively. After appropriate treatment, the samples were washed with 0.5% sodium azide, fixed with 1% paraformaldehyde, and analyzed by flow cytometry using a BD FACSCaliburTM as previously described (Jimenez Flores, Tian, Sizova, Epstein, Lamont, & Uriarte, 2017). The release of MMP-9 (a marker for gelatinase granule exocytosis) was measured in the neutrophil supernatants, collected from the experimental conditions described above, by MMP-9 ELISA (Boster) following the manufacturer's instructions; as well as the enzymatic activity analyzed by gelatin zymography assay as previously described (Jimenez Flores, Tian, Sizova, Epstein, Lamont, & Uriarte, 2017). The gels were stained with 0.5% Coomassie blue followed by subsequent destaining with 50% methanol. Densitometry of the 92 kDa band intensity was semi-quantified using ImageJ software.

Statistical Analysis

Statistical analysis were performed using one-way analysis of variance (ANOVA) and the Tukey-Kramer multiple-comparison post-test or paired two-tailed Student's *t*-test using GraphPad Prism Software. Differences were considered significant at the level $p < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. *F. alocis* engulfment by human neutrophils

Neutrophils were challenged with non-opsonized or serum opsonized Alexa Fluor 488 *S. aureus*, or non-opsonized or 10 % serum opsonized CFSE-labeled *F. alocis* for 30 min. After the 30 min (A-B) of bacterial challenge, cells were visualized and quantified via ImageStreamX flow cytometry. 1000 neutrophil events were collected and sorted into bacteria positive or negative bins based on CFSE / AlexaFluor 488 intensity and an internalization mask (Erode 4) designed to ignore signals from associated or extracellular bacteria was used for the analysis. The bacterial phagocytosis was calculated as an infection index, as described in material and methods. Data are expressed as the mean $\pm$ SEM of the infection index from 4 independent experiments. C. Representative confocal images of the WGA-stained neutrophil membrane and non-opsonized or 10% serum opsonized CFSE-labeled *F. alocis* after 30 min challenge. D. Percent of CFSE-positive neutrophils with attached/internalized (internalized), or attached only non-opsonized *F. alocis*. E. Percent of CFSE-positive neutrophils with attached/internalized (internalized), or attached only opsonized *F. alocis*. For D-E, Percent of data are expressed as mean $\pm$ SEM of percent of CFSE-positive neutrophils for 3 separate experiments. * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$. ns: non-significant.

Figure 2. *F. alocis* survival inside human neutrophils

Neutrophils were challenged with opsonized *F. alocis* (MOI 10, for 0.5-2-4 h). Viable (green) and non-viable (red/orange) *F. alocis* was distinguished using BacLight DNA dyes SYTO9 and propidium iodide (PI). **A.** Representative confocal image where white dashed arrows indicate viable *F. alocis* (green) and white arrows indicate non-viable (red/orange). N: neutrophil nucleus. **B.** The percent of viable internalized (attached to and internalized by neutrophils) *F. alocis* was quantified from 100 neutrophils, respectively. Data are expressed as the mean $\pm$ SEM of the percent of viable *F. alocis* attached to and internalized; from 3 independent experiments. **C.** TEM images of neutrophils challenged with opsonized *F. alocis* for 2-4-6-20 h. White dashed arrows indicate phagosomes containing electron dense *F. alocis* and white arrows indicate phagosomes containing electron-lucent bacteria. Magnification shown is 9800 x. ns= non-significant

Figure 3. *F. alocis* induced minimal respiratory burst response in human neutrophils

A. Neutrophils were unstimulated (Basal), or challenged with non-opsonized (*S. a*), opsonized *S. aureus* (OpS.a), non-opsonized *F. alocis* (0 % serum) or with increasing concentrations of serum opsonized *F. alocis* (2-5-10 % serum) for 10 or 30 min and intracellular respiratory burst response was analyzed by measuring the change of fluorescence after oxidation of dichlorofluorescein diacetate (DCF) by flow cytometry. Data are expressed as mean $\pm$ SEM of the mean channel of fluorescence (mcf) from 5 independent experiments. *** $p < 0.0001$ compared to basal, S.a, and *F. alocis* 0-2-5-10 (% serum) conditions. **B.** Neutrophils were unstimulated (Basal), or challenged with opsonized *S. aureus* (OpS.a), non-opsonized, 10% opsonized (Op) *F. alocis* ATCC38596 (ATCC), or low clinical passage strain D-62D (D62) for 10 or 30 min and the intracellular respiratory burst response was analyzed by flow cytometry as described in material and methods. **A.** Data are expressed as mean $\pm$ SEM of the mean channel of fluorescence (mcf) from 4 independent experiments. **C.** The intracellular production of ROS was monitored over time up to 180 min. Neutrophils were unstimulated (Basal), or stimulated with PMA, or non-opsonized *F. alocis* (Non op-*F. alocis*) or opsonized *F. alocis* (Op-*F. alocis*) following synchronized phagocytosis and the rate of ROS production reported every 10 min intervals. Data are expressed as the mean $\pm$ SEM of the change in DCF fluorescence per min from 5 independent experiments. $p < 0.0001$ for PMA vs Non-op or Op *F. alocis*. **D.** The NBT assay was used to determine the intracellular superoxide production by the presence of blue formazan deposits. Images were analyzed by light microscopy and confocal microscopy as display on the far right of the panel challenging neutrophils with 10% opsonized CFSE-labeled *F. alocis* (CFSE-Op-*F. alocis*). White arrows depict NBT-positive phagosomes and dashed arrows depict NBT negative phagosomes. **E.** Neutrophils were unchallenged (Basal),

stimulated with fMLF, or challenged with *F. alocis* for 5, 30 and 60 min. Following stimulation extracellular production of superoxide was measured by the colorimetric reduction of ferricytochrome c. Data are expressed as the mean $\pm$ SEM of $[\text{O}_2^-]$ nmol/ 4×10^6 cells released from 8 independent experiments. *** $p < 0.0001$. ns= non-significant.

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Figure 4. Neutrophils internalize live and heat killed *F. alocis* with similar efficiency

Neutrophils were challenged with live or heat killed (HK) opsonized CFSE-labelled *F. alocis* MOI 10 for 30 min. A-B, cells were visualized and quantified via ImageStreamX flow cytometry. 1000 neutrophil events were collected and sorted into bacteria positive or negative bins based on CFSE / AlexaFluor 488 intensity and an internalization mask designed using the WGA-stained neutrophil membrane to ignore signals from associated or extracellular bacteria. The bacterial phagocytosis was calculated as an infection index, as described in material and methods. Data are expressed as the mean $\pm$ SEM of the infection index from 7 independent experiments. C. Representative confocal image of the WGA-stained neutrophil membrane and 10% serum opsonized heat killed CFSE-labeled *F. alocis* (HK-*F. alocis*) after 30 min challenge. D. Percent of CFSE-positive neutrophils with attached/internalized (internalized), or attached only opsonized heat-killed (HK)-*F. alocis*. Percent of data are expressed as mean $\pm$ SEM of percent of CFSE-positive neutrophils for 3 separate experiments. * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$. ns: non-significant.

Figure 5. *F. alocis* does not inhibit ROS production to other particulate stimuli

A. Neutrophils were challenged with opsonized *S. aureus* (Op-*S. aureus*), or opsonized *F. alocis* (Op-*F. alocis*), or heat-killed opsonized *F. alocis* (Op-HK-*F. alocis*) for 30 min. Intracellular ROS production was determined by the change of fluorescence after oxidation of DCF by flow cytometry. Data are expressed as mean $\pm$ SEM of the mean channel of fluorescence (mcf) from 5 independent experiments. **B.** Neutrophils were unstimulated (Basal), or challenged with opsonized *F. alocis* (Op-*F. alocis*) or heat-killed opsonized *F. alocis* (Op-HK-*F. alocis*) following synchronized phagocytosis and the rate of ROS production induced by oxidation of DCF for 180 min. Data are expressed as the mean $\pm$ SEM of the change of fluorescence per min from 5 independent experiments. $p < 0.05$ for Op-HK-*F. alocis* vs Op-*F. alocis*. **C.** Neutrophils were exposed to *F. alocis* culture supernatant (Fa sup) alone, or challenged with *S. aureus* (30 min), or pre-treated with *F. alocis* culture supernatant for 15 min followed by *S. aureus* challenge (Fa sup + *S. aureus*; 30 min) and the intracellular ROS production was determined flow cytometry as described above. Data are expressed as mean $\pm$ SEM of the mean channel of fluorescence (mcf) from 4 independent experiments. **D.** Neutrophils were challenged with *S. aureus* (Alone, MOI 10, for 30 min), or pre-treated with *F. alocis* (F.a, MOI 10, for 15 min) followed by *S. aureus* (MOI 10, for 30 min), or pre-treated with *S. aureus* (S.a, MOI 10, for 15 min) followed by *S. aureus* (MOI 10, 30 min), or co-infected with *F. alocis* and *S. aureus* (F.a + S.a, each organism at MOI 5, for 30 min), or *F. alocis* alone (F. a, MOI 10, for 30 min). Intracellular ROS production was measured by flow cytometry as described above. Data are expressed as mean $\pm$ SEM of the mean channel of fluorescence (mcf) from 6 independent experiments. * $p < 0.05$ compared to alone; *** $p < 0.0001$ compared to all the experimental conditions. ns= non-significant. **E.** Neutrophils were challenged with *P. stomatis* (MOI 10), or co-infected

with *F. alocis* and *P. stomatis* (F.a + P.s; each organism at MOI 10), or *F. alocis* (MOI 10) for 30 min. Intracellular ROS production was measured by flow cytometry as described above. Data are expressed as mean $\pm$ SEM of the mean channel of fluorescence (mcf) from 5 independent experiments. * $p < 0.05$ compared to compared to all the experimental conditions. ns= non-significant.

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Figure 6. *F. alocis* prevents specific granule recruitment to its phagosomes

Neutrophils were unstimulated, or challenged with AlexaFluor 488-labeled opsonized (Op)-*S. aureus*, CFSE-labelled heat-killed (Op-HK) *F. alocis*, or CFSE-labelled opsonized (Op) *F. alocis*, MOI 10 for 30 min. **A.** Specific granule recruitment to bacteria-containing phagosomes was visualized by confocal microscopy. White arrows indicate lactoferrin positive enrichment to bacteria-containing phagosomes, and white dashed arrows indicate lactoferrin negative enrichment to bacteria-containing phagosomes. **B.** Approximately 100 infected cells per condition were examined and phagosomes were labeled as lactoferrin positive if 50% of the phagosome was surrounded by the granule marker. Data are expressed as the mean ± SEM of the percent of lactoferrin positive phagosomes from 3 independent experiments. DAPI (depicted in blue) was used to stain the neutrophil nucleus. **p<0.001.

Figure 7. *F. alocis* prevents azurophil granule recruitment to its phagosomes

Neutrophils were unstimulated, or challenged with AlexaFluor 488-labeled opsonized (Op)-*S. aureus*, CFSE-labelled heat-killed (Op-HK) *F. alocis*, or CFSE-labelled opsonized (Op) *F. alocis* for 30 min. **A.** Azurophil granule recruitment to bacteria-containing phagosomes was visualized by confocal microscopy. Neutrophil elastase was used as a marker to determine azurophil granule recruitment to bacteria-containing phagosomes. White arrows indicate neutrophil elastase positive enrichment to bacterial containing phagosomes, and white dashed arrows indicate neutrophil elastase negative enrichment to bacteria-containing phagosomes. **B.** Approximately 100 infected cells per condition were examined and phagosomes were labeled as neutrophil elastase positive if 50% of the phagosome was surrounded by the granule marker. Data are expressed as the mean ± SEM of the percent of neutrophil elastase positive phagosomes from 3 independent experiments. DAPI (depicted in blue) was used to stain the neutrophil nucleus. * $p < 0.05$, ** $p < 0.001$.

Figure 8. Opsonized *F. alocis* induces exocytosis of three of the four neutrophil granule subtypes Neutrophils were unchallenged (basal), challenged with fMLF (5 min), or challenged with live or heat killed (HK) opsonized *F. alocis* (MOI 10, 30 min). **A-B.** Secretory vesicle and specific granule exocytosis was determined via flow cytometry by measuring the increase in plasma membrane expression of CD35 or CD66b respectively. Data are expressed as the mean $\pm$ SEM of the mean channel of fluorescence (mcf) from 6 independent experiments. In **A**, ns: non-significant compared to basal. **C.** Gelatinase granule exocytosis was measured by ELISA as levels of matrix metalloproteinase 9 (MMP-9) present in the supernatants collected from the different experimental conditions. Data are expressed as mean $\pm$ SEM of MMP-9 release in ng/4 $\times$ 10⁶ cells, from 5 independent experiments. **D.** Gelatin zymography gels were used to determine the activity of MMP-9 in the supernatants from the same experimental conditions described above. The protein standard is shown on the left side, and the different MMP-9 isoforms are also indicated. **E.** Quantification of the densitometry values of the gel degradation corresponding to the 92 kDa band was analyzed by ImageJ software, from the gels of 4 independent experiments. **F.** Azurophil granule exocytosis was determined by the increase in plasma membrane expression of the CD63 marker by flow cytometry. Data are expressed as the mean $\pm$ SEM of the mean channel of fluorescence (mcf) from 5 independent experiments. *p<0.05, ** p<0.001, ***p<0.0001. ns= non-significant.