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The emerging oral pathogen, Filifactor alocis, modulates antimicrobial responses in primed human neutrophils.

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By

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Abstract

Periodontitis is an irreversible, chronic inflammatory, infectious disease of the oral cavity that affects approximately half of all adults 30 years or older in the USA. The oral cavity is under high immune surveillance because of its constant exposure to microbes in the environment. The primary immune cell responsible for this surveillance is the neutrophil. Pathogens associated with periodontitis possess virulence factors and have evolved strategies to evade neutrophil antimicrobial responses to survive. One such pathogen is *Filifactor alocis,* whose presence is associated with the progression of periodontitis. *F. alocis* manipulates several neutrophil antimicrobial functional responses to avoid killing, an evasion strategy that might perpetuate the unresolved destructive inflammation that is the hallmark of periodontitis.

An essential aspect of the host immune response against a threat is the release of cytokines and chemokines, a wide array of small endogenous inflammatory mediators that communicate between cells and evoke different responses from the cells, contributing to the initiation and resolution of the inflammatory response. Specific inflammatory cytokines and chemokines are associated with inflammation of the periodontium, a specialized tissue that surrounds and supports the tooth structure. These inflammatory mediators can prime or preactivate quiescent neutrophils. A primed neutrophil will display an enhanced response to a secondary stimulus compared to a quiescent or unprimed cell. Besides endogenous priming agents, exogenous agents such as the lipopolysaccharide (LPS) from bacteria can prime neutrophils via pattern recognition toll-like receptors (TLRs).

This project aims to define if *F. alocis* modulates the antimicrobial responses of primed neutrophils to promote its survival and the progression of periodontitis. In this project, two endogenous priming agents, tumor necrosis (TNF)α and interleukin (IL)-8, and LPS as an

exogenous priming agent, were tested. LPS stimulates TLR4, but if the preparation contains additional bacterial components like lipoproteins, it also stimulates TLR2. To assess the response of LPS-primed neutrophils toward *F. alcois,* three sources of LPS were used as priming agents for this project: *Escherichia coli* LPS-EK (containing lipoproteins), *E. coli* LPS-EK Ultrapure (no lipoproteins), and LPS from the keystone periodontal pathogen, *Porphyromonas gingivalis*. The data showed that *F. alocis* internalization was significantly increased in TNFα and both forms of tested *E. coli* LPS primed neutrophils compared to unprimed cells, both by flow cytometry and confocal microscopy.

In contrast, phagocytosis of *F. alocis* was similar between unprimed or IL-8 primed neutrophils. Interestingly, the respiratory burst response elicited by *F. alocis* was similar between unprimed and neutrophils primed by TNFα, IL-8, or *E. coli* LPS. However, *P. gingivalis* LPS was unable to prime neutrophils. These findings suggest that although TNFα and LPS primed neutrophils have enhanced phagocytosis towards *F. alocis*, it does not result in enhanced respiratory burst response. These results suggest that *F. alocis* can disable one of the phagocyte's critical antimicrobial functions, the respiratory burst response, to evade killing. Another exciting finding suggests that *F. alocis* might modulate the IL-8 signaling pathway to prevent internalization. Preliminary data also suggest that priming neutrophils with TNFα did not enhance the killing of *F. alocis*. In summary, the observed modulations of primed neutrophil antimicrobial responses by *F. alocis* may represent one of the organisms' evasion strategies to survive neutrophils in the hostile inflamed periodontal tissue.

Lay Summary

Periodontitis is an inflammatory disease of the gum tissue caused by certain oral pathogens' colonization of the gums. This disease is widespread, affecting hundreds of millions of people worldwide, and is associated with other systemic diseases such as diabetes and cardiovascular disease. Neutrophils are white blood cells of the immune system that patrol the mouth tissues in high numbers. Usually, neutrophils can effectively kill pathogens by employing several antimicrobial responses. However, pathogens associated with periodontitis, including the pathogen *Filifactor alocis*, can hijack these immune responses and harm the host by leading to tissue destruction and tooth loss. In the body, neutrophils can communicate with cells of the gingival tissue and are notified of infection by small proteins called cytokines. Exposure to cytokines causes an enhancement of their antimicrobial responses, a process known as "priming." Priming can also be induced by molecules derived from pathogens themselves. Previous characterizations of neutrophil interactions with *F. alocis* used unprimed cells. This project aimed to characterize the interactions of neutrophils primed with different priming agents with *F. alocis* to reflect more on what occurs in the body during infection. I found that *F. alocis* can modulate certain antimicrobial responses of primed neutrophils, which may contribute to its survival and the progression of periodontitis.

Introduction

Periodontitis is an irreversible, chronic inflammatory disease of the periodontium caused by microbial subversion of the host immune system [1]. Epidemiological studies have shown that this disease affects 20 – 50% of the global population and almost half of the USA adult population [2, 3]. The periodontium is the tissue that supports the teeth and acts as a barrier between the

microorganisms in the oral cavity and the underlying tissue structures beneath [4]. As a result, the oral cavity is constantly exposed to foreign materials, from the air we breathe to food intake. In health, accumulating indigenous microbes in the oral cavity establish a biofilm of symbiotic organisms that coexist harmoniously with the host immune system. However, colonization of the oral cavity by certain pathogenic microbes can alter the biofilm composition and break the homeostasis between the microbial community and the host [5]. Analysis of the oral plaque by advanced sequencing technology reveals the complexity of the microbial community and identifies newly dominant organisms associated with the onset and progression of periodontitis. Pathogens associated with periodontitis have virulence factors that allow them to evade the host immune response, leading to prolonged inflammation. This chronic dysregulated inflammation at the periodontium is the hallmark of periodontitis. Chronic inflammation results in tissue destruction, activation of immune cells, and bone resorption, which can eventually lead to tooth loss. Periodontitis is associated with other chronic systemic diseases such as diabetes, cardiovascular disease, and rheumatoid arthritis [6]. Periodontal pathogens benefit from the host's dysregulated inflammation by acquiring nutrients such as amino acids from the damaged tissue to support bacterial growth. This positive feedback loop benefits the pathogens' growth but to the detriment of the host [5].

Filifactor alocis is a Gram-positive, anaerobe, rod-shaped bacterium found in high numbers in periodontal disease sites compared to healthy sites [7]. *F. alocis* displays many interactions with other periodontal pathogens, such as *Porphyriomnas gingivalis* and *Aggregatibacter actinomycetemcomitans,* further complicating the dynamics of periodontitis and its progression [8]. *F. alocis* has several virulence factors that allow for its survival in the presence of immune cells and the progression of periodontitis (Fig 1, [9]). For example, *F. alocis* might

detoxify reactive oxygen species (ROS) generated by immune cells via FA796 and FA519 proteins via superoxide dismutase activity and hydrogen peroxide resistance, respectively [10, 11]. Extracellular vesicles released by *F. alocis* have also been shown to cause bone resorption of large bones associated with osteoporosis via osteoclastogenesis and inhibit complement-mediated opsonization via the *F. alocis* complement inhibitor (FACIN) protein [12, 13]. *F. alocis* may also directly contribute to the inflammation of the periodontium by evoking the release of pro-inflammatory cytokines from local gingival fibroblasts [14].

Figure 1

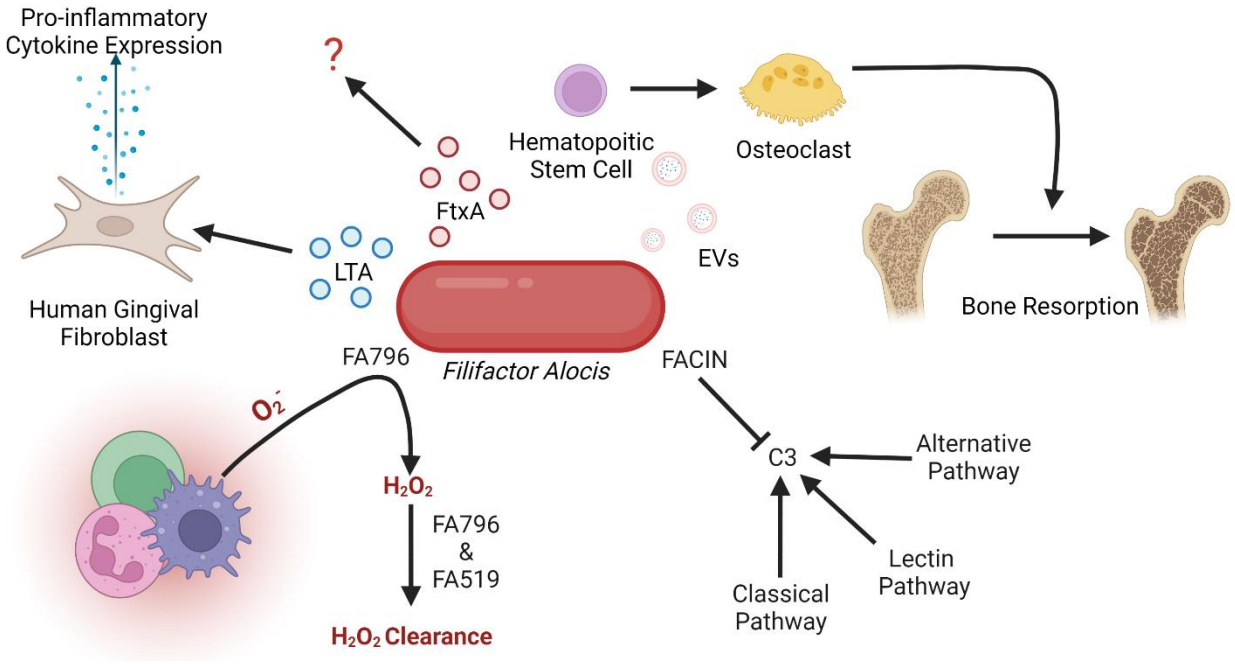


Fig 1. Summary of *F. alocis* virulence factors. Adapted from [9]. Oxidative resistance is achieved by converting superoxide generated by innate immune cells to H_2O_2 by FA796. The proteins FA796 and FA519 are associated with H_2O_2-induced stress resistance. *F. alocis* complement inhibitor (FACIN) binds complement component 3 (C3), essential to all three complement pathways. Extracellular vesicles (EVs) contain lipoproteins that stimulate osteoclastogenesis in committed osteoclast precursors via TLR2, contributing to bone resorption. The novel

RTX exotoxin, FtxA, is found in 60% of clinical isolates, but its biological effects are unknown. Lipoteichoic acid (LTA) induces the expression of pro-inflammatory cytokines by human gingival fibroblasts.

Polymorphonuclear leukocytes, known as neutrophils, are vital components of the innate immune system. A high number of neutrophils are present in the oral cavity. Their high prevalence in the oral cavity makes them the first to respond to infection in oral sites where they can deploy many antimicrobial responses. The gingival crevice is the space between the host tooth and gingiva where pathogens may accumulate in the resident biofilm. Gingival crevicular fluids may carry molecules associated with infection and host factors secreted in response to the infection to the bloodstream of the highly vascular gingiva, which contact and signal for the recruitment of neutrophils to the gingival crevice to respond to infection [15]. Antimicrobial responses such as phagocytosis, ROS production via activation of the respiratory burst response, granule exocytosis or fusion to the phagosome, and neutrophil extracellular traps (NETs) can all be deployed by neutrophils to subdue infection [16]. While these responses are usually limited to killing pathogens, they can cause collateral damage to host tissue. The Uriarte lab showed that neutrophil encounter with *F. alocis* results in minimal ROS production, impaired phagosome maturation, and no NET formation, allowing for ≥ 65% of the organism to remain viable 4 hours post-infection, demonstrating its effective evasion strategy [17]. Furthermore, *F. alocis* promotes the release of neutrophil-derived cytokines and chemokines, which might be how the organism sustains a pro-inflammatory environment in the oral mucosa [18].

An essential aspect of the neutrophil's response to infection is the state of priming these cells can acquire. Host-derived molecules, such as cytokines and chemokines, and pathogen-derived molecules, such as lipopolysaccharide (LPS), lipoteichoic acid (LTA), or peptidoglycan, can prime neutrophils [19]. This intermediate primed state causes phenotypic changes in the

magnitude of neutrophil response towards a stimulus compared to quiescent/resting cells. For example, primed neutrophils mount an enhanced response to a stimulus compared to the response elicited by non-primed cells to that same stimulus [19]. Interest in primed neutrophils is increasing as they have been found in the circulation of patients with diseases correlated with periodontitis, such as diabetes [20]. Tumor necrosis factor(TNF)α and interleukin(IL)-8 are potent host-derived inflammatory molecules that can prime neutrophils. Both inflammatory mediators are elevated in periodontitis patients [21]. Neutrophils have specific surface receptors that recognize each of these two priming agents leading to different signal transduction pathways. LPS is another potent priming agent in periodontitis sites shed by Gram-negative bacteria. For example, *P. gingivalis,* a Gram-negative periodontal pathogen, is strongly associated with the onset of periodontitis [22]. Blood from periodontitis patients contains *P. gingivalis* LPS, which could prime circulating neutrophils. Those primed neutrophils will encounter *F. alocis* once they reach the gingival crevice. Published *in vitro* experiments have characterized interactions between neutrophils and *F. alocis*; however, a knowledge gap exists in the response of primed neutrophils to *F. alocis*.

My working hypothesis is that *F. alocis* can modulate the antimicrobial responses of primed human neutrophils. To address this hypothesis, I assessed differentially primed neutrophils' phagocytosis and respiratory burst response when challenged with *F. alocis*. Furthermore, I began to characterize the overall killing ability of primed neutrophils toward *F. alocis*.

Materials and Methods

a.) Human neutrophil isolation.

Neutrophils were isolated by Dr. Uriarte's senior lab technician, Mario Gutierrez Lau, from healthy human donors utilizing the plasma-Percoll gradients as previously described [23]. Under microscopic evaluation via Wright staining, isolated cells will be shown to be >95% neutrophils for each used experiment. The study is approved and follows the guidelines set by the Institutional Review Board at the University of Louisville.

b.) Bacterial strains and growth conditions.

Dr. Aruna Vashishta, a senior research associate in Dr. Uriarte's lab, cultured *F. alocis* (ATCC 38596) as previously described [24, 25]. Heat-killed *F. alocis* was generated by 60°C incubation for 60 minutes. UV-killed *F. alocis* was generated by exposing the bacteria to UV light for 30 minutes.

c.) Respiratory burst response.

To measure the intracellular respiratory burst response elicited by *F. alocis* challenge, neutrophils (2 x 10^6 cells/mL) were untreated, challenged with serum-opsonized *F. alocis* (ATCC38596; the multiplicity of infection (MOI) of 10 bacteria per neutrophil), serum-opsonized heat-killed *F. alocis* (MOI of 10), serum-opsonized UV-killed *F. alocis* (MOI of 10) for 30 minutes, or primed by TNFα (2ng/ml; 10 min, Abcam), followed by serum-opsonized *F. alocis*, by serum-opsonized heat-killed *F. alocis* (MOI of 10), by serum-opsonized UV-killed *F .alocis* (MOI of 10) for 30 minutes. Intracellular phagocytosis-induced ROS (icROS) was measured as a change of fluorescence after the oxidation of 2',7'-dichlorofluorescein (DCF) by BD FACS CaliburTM.

A kinetic intracellular ROS production assay was also performed. Neutrophils were resuspended in HBSS with Ca^{2+} and Mg^{2+} containing 25μM DCF (Molecular Probes) to 10^7 cells/ml for 20–25 min at room temperature with gentle agitation. Because this method was used to measure intracellular ROS production, the addition of the membrane-impermeable enzyme superoxide dismutase (SOD, 8.0μg, Sigma) to the reaction was necessary to remove extracellular superoxide or hydrogen peroxide and allow measurement of ROS generated only in intracellular compartments. Each well contained unprimed neutrophils (1 x 10^6 cells/ml) or primed with TNFα (2ng/ml; 10 min), IL-8 (10nM; 60 min, R&D Systems), LPS (100ng/mL, 60 min); following the priming time neutrophils were challenged with opsonized-*F. alocis* (MOI of 10), heat-killed *F. alocis* (MOI of 10), UV-killed opsonized-*F. alocis* (MOI of 10), or 0.2 μg of Phorbol 12-myristate 13-acetate (PMA), a potent inducer of ROS used as a positive control (Sigma). The three types of LPS were all used at the same priming concentration of 100ng/ml for 60 min pre-treatment. The Invivogen LPS is ultrapure from *E. coli*, which is recognized only by TLR4. Another Invivogen LPS from *E. coli* contains lipoproteins that allow this agonist to signal both through TLR2 and TLR4. The third LPS molecule is derived from the periodontal pathogen *P. gingivalis* and is antagonistic to TLR4 (Sigma). To synchronize phagocytosis cells in a 96-well microtiter plate were kept at 14°C, centrifuged for 4 min at 600×g, and transferred to a micro-plate 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 538nm, respectively. The rate of neutrophil ROS production over time was determined from the average fluorescence for triplicate wells within each 10 min period.

d.) Phagocytic ability of *F. alcois*-challenged neutrophils.

Human neutrophils primed with TNFα (2ng/ml; 10 min), IL-8 (10nM; 30 min), LPS (100ng/mL, 60 min), or unprimed cells (4×10^6 cells/ml) were challenged with carboxyfluorescein succinimidyl ester (CFSE)-labelled opsonized-*F. alocis* (MOI of 10), heat-killed *F. alocis* (MOI of 10), or UV-killed opsonized-*F. alocis* (MOI of 10). Cells were incubated in a shaking water bath at 37°C for 30 and 60 min, pelleted at 6,000g for 30 sec, and rinsed with hanks balanced solution (HBSS, Thermo Fisher) with Ca^{2+} and Mg^{2+} and fixed with 1% paraformaldehyde. The neutrophil plasma membrane was stained for 10 min wheat germ agglutinin (WGA, Invitrogen). Images were obtained, quantified, and visualized using an Amnis ImageStreamX (Millipore). Labeled bacterial internalization was detected using a 488-nm solid-state laser, and WGA staining was detected using a 642-nm solid-state laser. One thousand 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 for counting the CFSE or Alexa Fluor 488 positive cells and ignoring positive signals from membrane-associated or extracellular bacteria. The Erode mask (Erode 4) was created based on the Ideas software user manual to remove the selected number of pixels from all edges of the starting mask.

Phagocytosis was also assessed by confocal microscopy. Human neutrophils primed with TNFα (2ng/ml; 10 min), IL-8 (10nM; 60 min), LPS (100ng/mL, 60 min), or unprimed cells ($4x\ 10^6$ cells/ml) were suspended in RPMI+10% FBS and placed on acid-washed coverslips coated in a 1:1 ratio of human serum (Sigma S7023) and sterile 1XPBS containing Ca^{2+} and Mg^{2+}. The cells were incubated for 30 min to 1 hr at 37 ºC to allow for settling onto the coverslip. CFSE-labelled opsonized-*F. alocis* (MOI of 10), heat-killed *F. alocis* (MOI of 10), or UV-killed opsonized-*F.*

alocis (MOI of 10) were distributed evenly over the cell solution. The plate was centrifuged for 4 min at 600xg at 14 ˚C to synchronize phagocytosis. Supernatants were aspirated, and cells were fixed cells using 4% PFA for 10 min. Supernatants were aspirated, and 200 µl of 3% BSA in PBS was added to each well for blocking. Plates were then incubated at room temperature for 1 hr on a slow rocker (if necessary, left at 4 ˚C overnight), and the blocking solution was removed by aspiration. To stain the extracellular *F. alocis*, 200 µl of rabbit polyclonal anti-*Fa* (1µg/ml) primary antibody in 3% BSA-PBS was added to each well and incubated at room temperature for 1 hr on a slow rocker. Coverslips were washed three times with 1X PBS, then 200µl of goat anti-rabbit Alexa Fluor 647 (Cell Signaling) secondary antibody in 3% BSA-PBS was added to each well. Plates were then incubated at room temperature for 1 hr on a slow rocker, followed by two washes with 1X PBS. Antibody linkages were fixed by adding 200µl of 4% PFA and incubating for 10 min. For nuclear staining, DAPI was used at a final concentration of 300 nM for 5 min and was washed twice with 500µl 1X PBS for 5 min each at room temperature. Fixed coverslips were then mounted with 4µl of Prolong Gold Antifade reagent (Invitrogen). The edges of the coverslips were sealed with clear nail polish. Slides were visualized using the Leica TCS SP8 MP Confocal Microscope. Image analysis was done using LAS X software. Quantification was performed by counting the total internalized and outside bacteria both from approximately 50-150 neutrophils from 10 visual fields of each coverslip.

e.) Killing ability of *F. alocis* by neutrophils.

The bacterial killing was assessed by surveying the ability of *F. alocis* to form colony-forming units (CFU) after the neutrophil challenge. Human neutrophils primed with TNFα (2ng/ml; 10 min) or unprimed cells (2×10^6 cells/ml) and resuspended in RPMI + 5% FBS, were then challenged with serum-opsonized *F. alocis* (MOI of 10) and placed in an incubator for 1 hr. Post

challenge, neutrophils were centrifuged at 4 ˚C for 5 minutes at 100xg. The supernatant was collected, and the resulting pellet was washed 950µL of endotoxin-free PBS (Millipore), collecting the supernatants each time and pooling them. Next, the pellet was resuspended in 100µL 0.5% saponin in PBS (Sigma). The supernatant pool was centrifuged at 4 ˚C for 5 minutes at 100xg and resuspended in 100µL of endotoxin-free PBS. The solutions were then diluted, and 10µL of each sample were plated. The plates were allowed to dry near a Bunsen burner for 5-10 min and placed in an anaerobic jar for 6-7 days.

f.) Statistical analysis.

All statistical examinations were performed using a one-way analysis of variance (ANOVA) alongside the Tukey-Kramer multiple-comparison test (GraphPad Prism Software, San Diego, CA, USA). Differences with a P-value less than 0.05 were determined to be statistically significant.

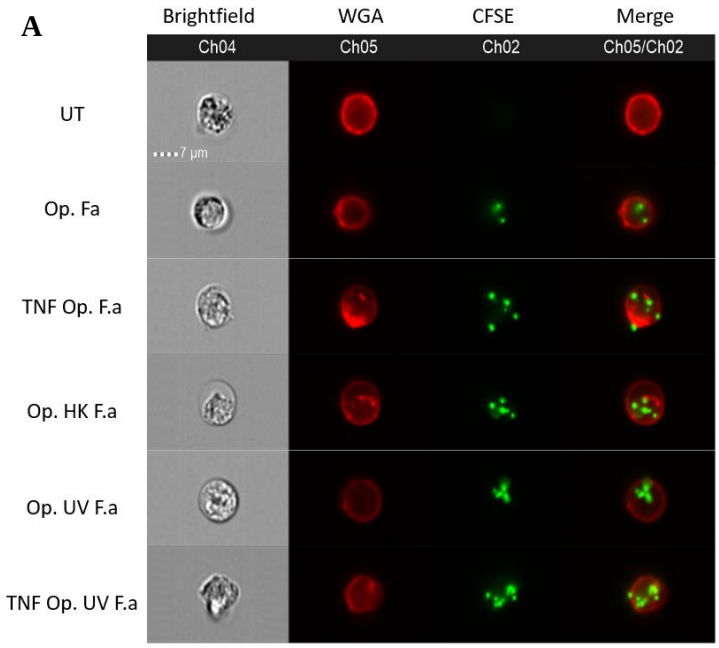
Results

F. alocis differentially modulates the phagocytic ability of primed neutrophils.

As neutrophils journey to the site of infection, chemical gradients composed of cytokines and chemokines released by cells in the proximity of infection and bacterial-derived products guide neutrophils to the infection. In addition, these priming agents preactivate neutrophils, enhancing the cell response when encountering the pathogen. Some of these cytokines and chemokines are prevalent in periodontitis, including TNFα and IL-8 [21]. Furthermore, bacteria-derived products such as LPS shed from gram-negative bacteria, are found in the bloodstream of patients with periodontitis [19].

To characterize if priming will enhance neutrophils' phagocytosis towards *F. alocis*, I employed a flow cytometric assay, and compared bacterial internalization between unprimed and TNFα primed neutrophils (Fig 2A). The data showed a significantly enhanced internalization of *F. alocis* upon TNFα priming at 30 and 60 min post-infection (Fig 2B). High internalization of heat-killed *F. alocis*, close to 80%, was seen in unprimed neutrophils, which was not further enhanced by TNFα priming (Fig 2A-B). Internalization of live and UV-killed *F. alocis* was similar in unprimed neutrophils and significantly enhanced with TNFα priming at 30 min post-infection (Fig 2B). There is no significant difference at the 60 min time of UV-killed *F. alocis* challenge by TNFα primed versus non-primed neutrophils; however, there appears to be a trend of similar means between the two groups in the two time points. Increasing the number of biological replicates may eventually show a significant difference at 60 minutes. This data indicates that TNFα priming enhanced phagocytosis of *F. alocis* by neutrophils.

Figure 2



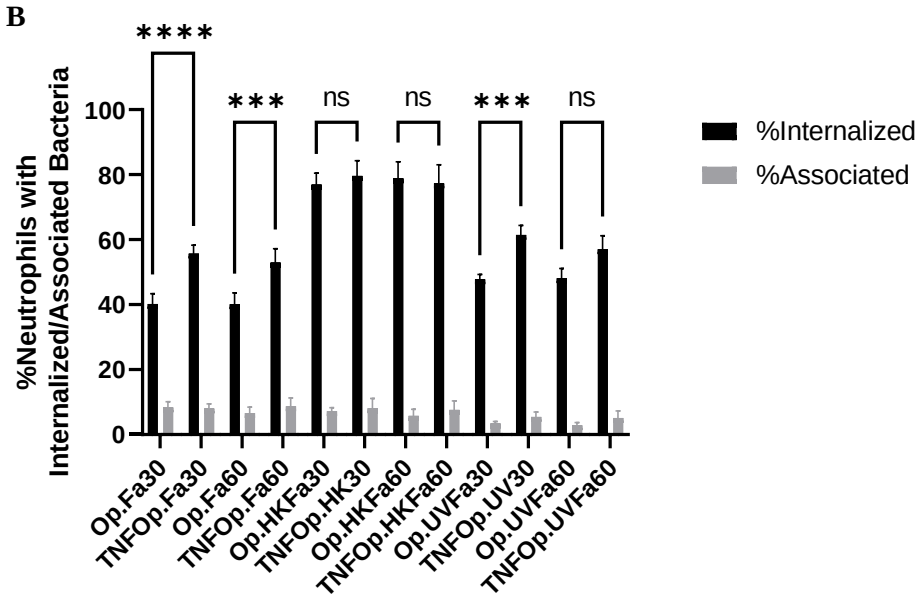


Fig 2. Effect of TNF*α* priming on phagocytosis of serum opsonized *F. alocis* by human neutrophils. Neutrophils ($4x10^6$ cells/mL) were unprimed or primed with TNFα (2ng/ml; 10 min), followed by challenge with serum opsonized *F. alocis* (Op.Fa, ATCC 38596), serum opsonized heat-killed *F. alocis* (Op.HKFa), serum opsonized UV-killed *F. alocis* (Op.UVFa), for 30 or 60 min. All bacterial conditions were done at a multiplicity of infection (MOI) of 10. Cells were visualized and quantified via ImageStreamX flow cytometry. One thousand neutrophil events were collected and sorted into bacteria positive or negative bins based on carboxyfluorescein succinimidyl ester (CFSE)/AlexaFluor 488 intensity and wheat germ agglutinin (WGA)-stained neutrophil membrane. (a) Representative flow cytometry images showing brightfield, neutrophil membrane staining (WGA), bacteria staining (CFSE), and merge images. (b) The bacterial phagocytosis was calculated as a percentage of cells that were CFSE-positive, both fully internalized and attached to the plasma membrane. Data are expressed as the mean ± standard error of the mean of the percentage. Mixed effect analysis, Sidak's multiple comparisons, n=8. ns = non-significant, *** = $p < 0.001$, **** = $p < 0.0001$.

Next, I sought to test the effects of IL-8 as a priming agent on the internalization of *F. alocis* by neutrophils. IL-8 primed neutrophils showed similar internalization of *F. alocis* compared to unprimed cells (Fig 3A, B). Furthermore, phagocytosis of heat-killed or UV-killed *F.*

alocis was similar in unprimed or IL-8-primed neutrophils (Fig 3B). These results suggest that *F. alcois* may be able to modulate the phagocytic response of neutrophils when primed by IL-8.

Figure 3

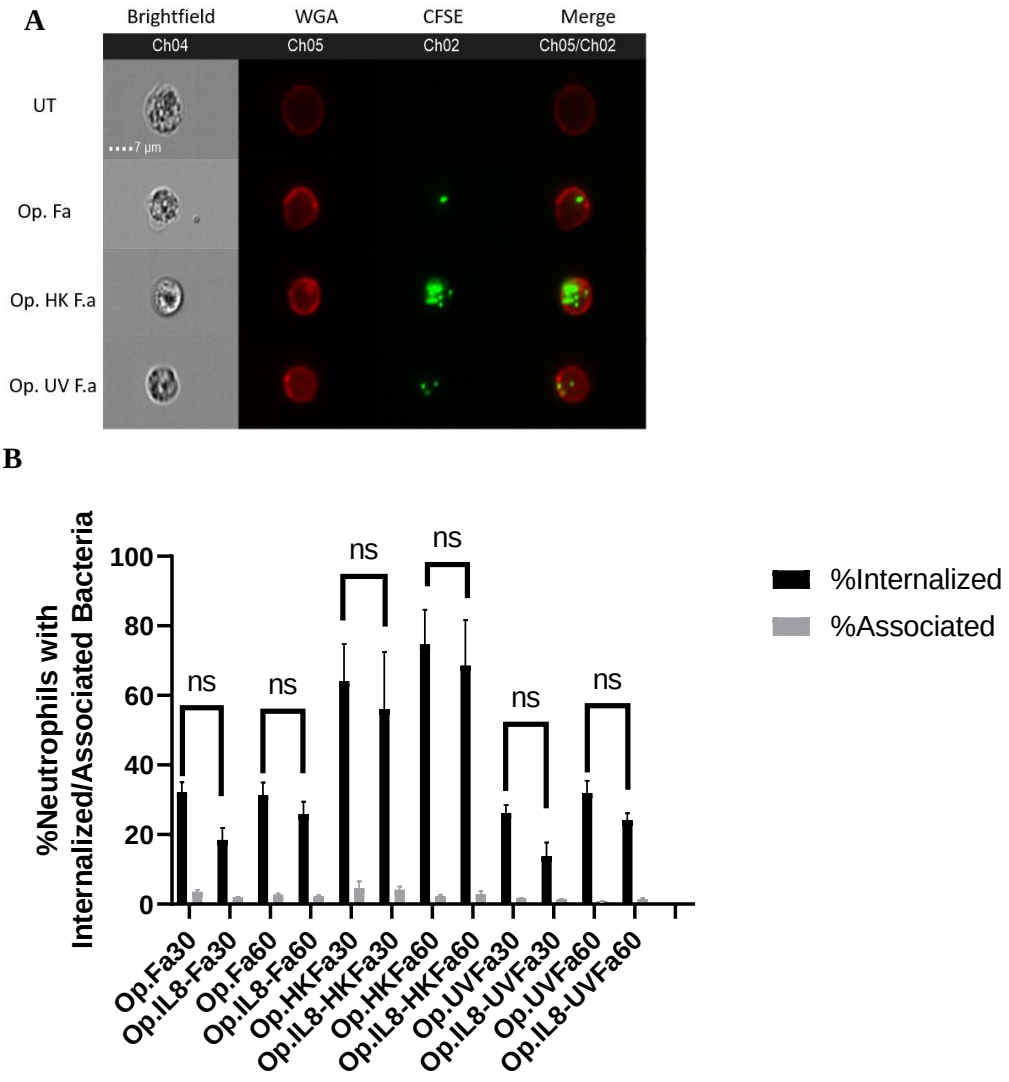


Fig 3. Effect of IL-8 priming on phagocytosis of serum opsonized *F. alocis* by human neutrophils. Neutrophils ($4x10^6$ cells/mL) were unprimed or primed with IL-8 (10nM; 60 min), followed by challenge with serum opsonized *F. alocis* (Op.Fa, ATCC 38596), serum opsonized heat-killed *F. alocis* (Op.HKFa), serum opsonized UV-killed *F. alocis* (Op.UVFa), for 30 or 60 min. All bacterial conditions were done at a multiplicity of infection (MOI) of 10. Cells were visualized and quantified via ImageStreamX flow cytometry. One thousand neutrophil events were

collected and sorted into bacteria positive or negative bins based on carboxyfluorescein succinimidyl ester (CFSE)/AlexaFluor 488 intensity and wheat germ agglutinin (WGA)-stained neutrophil membrane. (a) Representative flow cytometry images showing brightfield, neutrophil membrane staining (WGA), bacteria staining (CFSE), and merge images. (b) The bacterial phagocytosis was calculated as a percentage of cells that were CFSE-positive, both fully internalized and attached to the membrane. Data are expressed as the mean ± standard error of the mean of the percentage. One way ANOVA, n=3. ns = non-significant.

A confocal microscopy assay was performed to complement the data acquired by imaging flow cytometry to assess *F. alocis* internalization by neutrophils. The confocal assay differs from imaging flow cytometry in that neutrophils are attached to a coated coverslip instead of in suspension, and bacterial phagocytosis is synchronized. This confocal experimental setting favors bacterial internalization by neutrophils. Similar to the imaging flow cytometry, IL-8 priming did not result in enhanced phagocytosis of *F. alocis* (Fig 4). However, phagocytosis of heat-killed *F. alocis* was higher compared to live or UV-killed bacteria independently of priming. (Fig 4B-C-D).

Figure 4

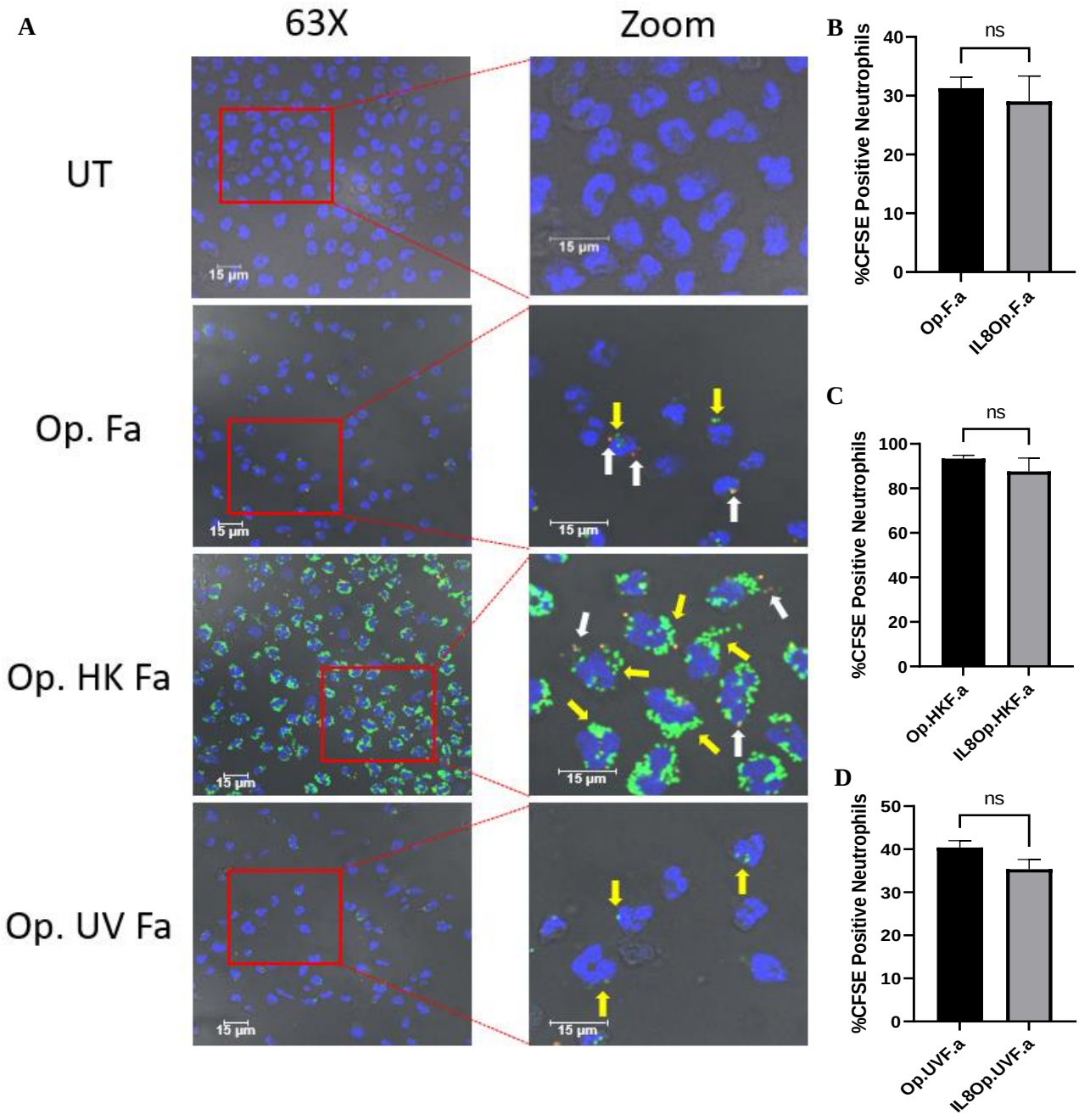


Fig 4. The effect of IL-8 priming on phagocytosis of serum opsonized *F. alocis* by human neutrophils. Human neutrophils primed with IL-8 (10nM; 60 min) or unprimed cells (4x 10^6 cells/ml) were challenged with CFSE-labelled opsonized-*F. alocis* (ATCC38596; MOI of 10), heat-killed *F. alocis* (MOI of 10), or UV-killed opsonized-*F. alocis* (MOI of 10). Slides were visualized using the Leica TCS SP8 MP Confocal Microscope and image

analysis was done using LAS X software.. (a) Representative confocal image showing nuclear staining (DAPI, blue), bacteria labeling (CFSE, green), extracellular bacterial labeling (Alexa Fluor 649. red), and a merged image where yellow arrows indicate examples of intracellular bacteria and white arrows represent examples of extracellular bacteria. (b-d) 50-150 neutrophils from 10 visual fields of each coverslip were counted and the percent of CFSE positive cells were quantified. Data are expressed as the mean ± standard error of percentage of CFSE + neutrophils from three independent experiments. One way ANOVA and Tukey posttest performed for each bacterial condition, n=3. ns = non-significant.

Bacterial derived molecules, such as LPS, are also potent neutrophil priming agents and highly abundant in the periodontium of periodontitis patients. LPS is a TLR4 ligand, but some LPS preparations contain other lipoproteins resulting in the activation of TLR2 as well. The periodontal pathogen, *P. gingivalis* modifies its LPS structure to blunt the TLR4 response. Next, I tested if using LPS from *E. coli* and *P. gingivalis* as a priming agent would enhance *F. alocis* phagocytosis by neutrophils. Neutrophils primed with the impure and purified *E. coli* LPS showed a significantly higher internalization of *F. alocis* than unprimed cells (Fig 5A and B). No significant difference was found between the internalization of *F. alocis* by unprimed cells and by *P. gingivalis*-LPS-primed cells providing further evidence that *P. gingivalis* LPS did not prime neutrophils (Fig 5C). Phagocytosis of heat-killed *F. alocis* by unprimed neutrophils is very high, close to 85%, which was not further enhanced by priming. UV-killed *F. alocis* internalization was higher in these experiments than those performed using IL-8 (≈ 80% vs. ≈40%, respectively). This difference was found to be attributed to a change in the media used to culture *F. alcois*. Overall, these results suggest that priming neutrophils with *E. coli* LPS enhanced the phagocytosis of *F. alocis*.

Figure 5

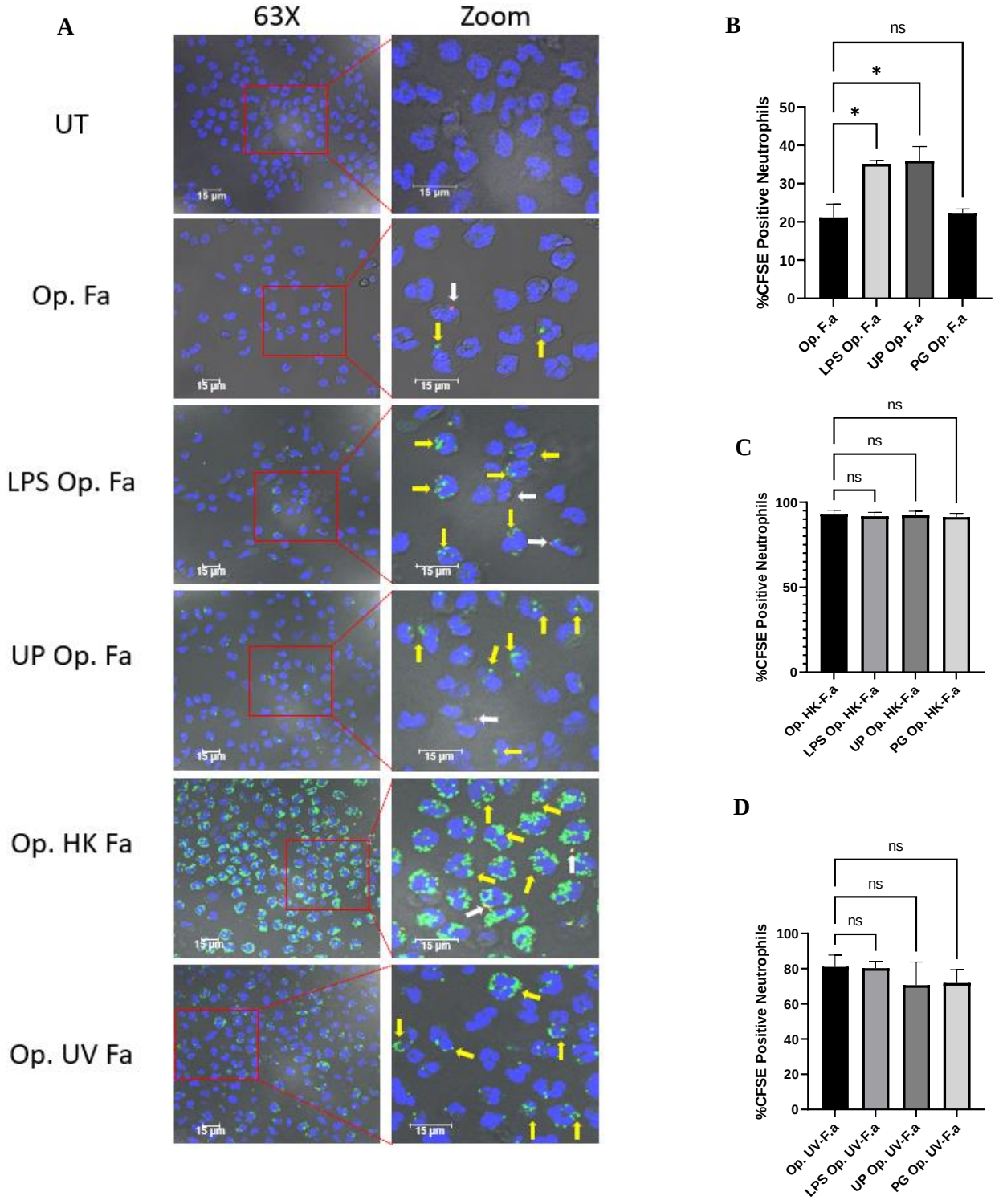

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Fig 5. The effect of LPS priming on phagocytosis of serum opsonized, attached *F. alocis* by human neutrophils. Human neutrophils primed with *E. coli* LPS (LPS), ultrapure *E. coli* LPS (UPLPS), or *P. gingivalis* LPS (PgLPS) (10ng/mL; 60 min) or unprimed cells (4x 10^6 cells/ml) were challenged with CFSE-labelled opsonized-*F. alocis* (ATCC38596; MOI of 10), heat-killed *F. alocis* (MOI of 10), or UV-killed opsonized-*F. alocis* (MOI of 10). Slides were visualized using the Leica TCS SP8 MP Confocal Microscope. (a) Representative confocal image showing nuclear staining (DAPI), bacteria labeling (CFSE), extracellular bacterial labeling (Alexa Fluor 649), and a merge image where yellow arrows indicate examples of intracellular bacteria and white arrows represent examples of extracellular bacteria. (b-d) Image analysis was done using LAS X software. Quantification was performed by counting the total internalized and outside bacteria from approximately 50-150 neutrophils from 10 visual fields of each coverslip. Data are expressed as the mean ± standard error of the mean of the percentage of three independent experiments. One way ANOVA and Tukey posttest were performed for each bacterial condition, n=3. * $p<0.05$; ns=non-significant.

F. alocis modulate the respiratory burst response of primed human neutrophils.

Upon phagocytosis of a particulate stimulus, like a bacterium, neutrophils mount a robust respiratory burst response with high ROS generation due to activating the NADPH oxidase complex assembled at the phagosomal membrane. However, *F. alocis* interaction with unprimed neutrophils results in minimal intracellular ROS (icROS) generation within the phagosome [17].

Neutrophil priming typically enhances neutrophil functional responses, including the respiratory burst response, increasing ROS production upon stimulation [2]. My results showed that phagocytosis of *F. alocis* was significantly enhanced when neutrophils were primed with TNFα and LPS from *E. coli*. Therefore, I hypothesized that *F. alocis* would induce minimal icROS production in IL-8-primed neutrophils but could not modulate the response in TNFα and *E. coli* LPS priming conditions. Intracellular ROS (icROS) production was measured to test the hypothesis. Since a significant increase in *F. alocis* phagocytosis was observed in TNFα primed neutrophils after 30 min of bacterial interaction (Fig 2), first, I determined the icROS production

induced by *F. alocis* upon encountering unprimed, or TNFα primed neutrophils at 30 min post-infection. Flow cytometry data showed no significant increase in icROS produced by TNFα primed neutrophils after 30 min of *F. alocis* challenge compared to the unprimed cells (Fig 6).

In contrast, TNFα-primed neutrophils challenged with heat-killed or UV-killed *F. alocis* showed a significantly enhanced respiratory burst response compared to unprimed conditions (Fig 6). Heat treatment will denature the bacterial surface components, whereas the surface components of UV-killed bacteria remain intact in ultraviolet treatment. Although the respiratory burst response induced by heat-killed and UV-killed *F. alocis* was significantly enhanced in TNFα primed neutrophils, the response elicited by the heat-killed organism, both in primed and unprimed conditions, was significantly higher compared to the response by the UV-killed bacterium (Fig 6). These findings suggest that live *F. alocis* can modulate the respiratory burst response of primed neutrophils. Furthermore, a surface component of *F. alocis* might be involved in the modulation of the respiratory burst response that subsequentially loses function upon the heat-killing of the organism.

Figure 6

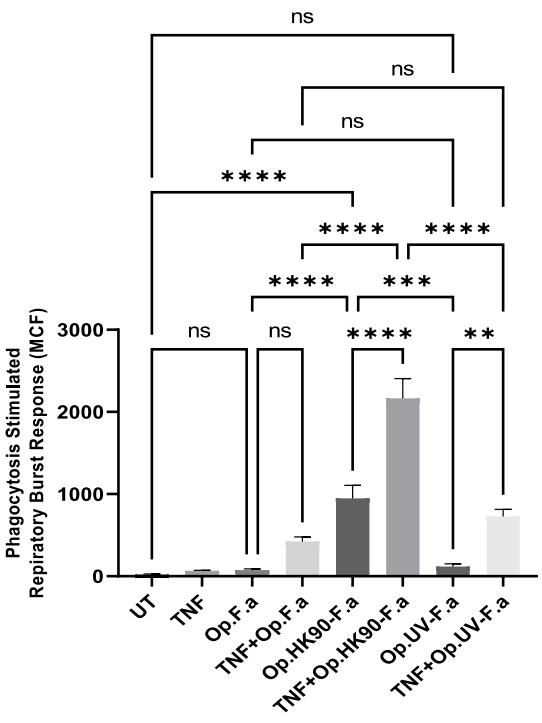


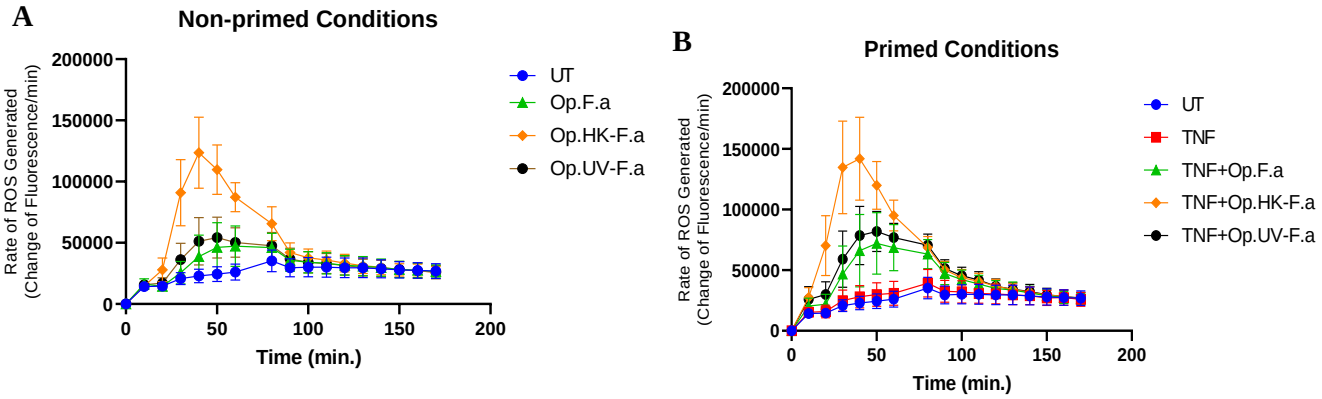
Fig 6. Viable *F. alocis* modulates the respiratory burst response of TNFα primed human neutrophils. Neutrophils ($2x10^6$ cells/mL) were untreated (UT), challenged with a multiplicity of infection (MOI) of 10 bacteria per neutrophil with serum opsonized *F. alocis* (Op. F.a), serum opsonized heat-killed *F. alocis* (Op.HK-F. a,), serum opsonized UV-killed *F. alocis* (UV-F. a) in the absence or presence of TNFα (2ng/ml; 10 min). Intracellular respiratory burst was measured by flow cytometry after 30 min of bacterial infection. The data are expressed as mean ± SEM of the mean fluorescence intensity (MFI). One way ANOVA and Tukey posttest, n=5. **** $p<0.0001$; *** $p<0.001$; ** $p<0.01$; ns=non-significant.

Because the infection is a dynamic process, I sought to understand how neutrophils responded to *F. alocis* throughout infection and how priming might change this response. To address this question, I performed a kinetic assay to measure the icROS production of neutrophils in response to *F. alocis* for 180 minutes. The data collected from a kinetic assay offers more detailed insights into the duration and peak of the respiratory burst response elicited by neutrophils

when interacting with a bacterium. Furthermore, the area under the curve generated by the kinetic assay can be calculated to reflect the total amount of icROS produced by the neutrophils during the infection. Comparing the amount of icROS generated by TNFα primed neutrophils to unprimed neutrophils, it is again apparent that *F. alocis* can modulate the respiratory burst response of primed neutrophils (Fig 7A-C).

Similarly to the results obtained 30 min post-infection (Fig 6), the UV-killed *F. alocis* cannot manipulate the primed respiratory burst response (Fig 7B and E). However, the amount of icROS produced in response to heat-killed *F. alocis* was not significantly increased upon priming (Fig 7B and D). This response might be due to heat-killed *F. alocis* already producing a high respiratory burst response in unprimed cells [17].

Figure 7



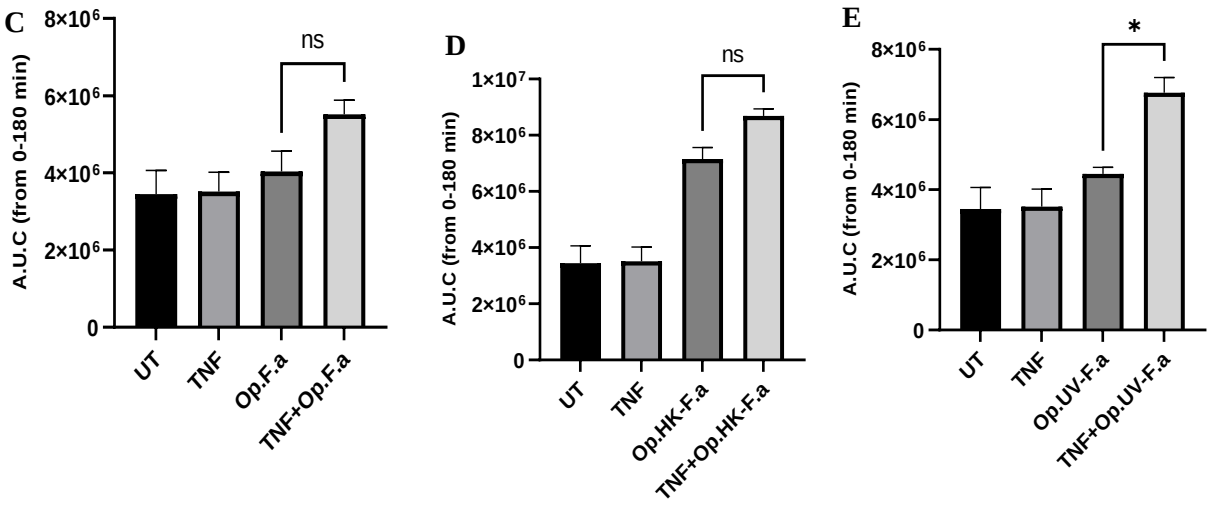


Fig 7. The effect of TNFα priming on the respiratory burst response induced by *F. alocis* in human neutrophils. The intracellular production of reactive oxygen species (ROS) was monitored over time up to 180 min. Neutrophils ($1x10^6$ cells/mL) were untreated (UT), incubated with TNFα (2ng/ml; 10 min), challenge with serum opsonized *F. alocis* (Op. F.a), serum opsonized heat-killed *F. alocis* (Op.HK-F. a), serum opsonized UV-killed *F. alocis* (UV-F. alocis), or TNFa followed by Op *F. alocis* (TNF+ Op. F.a), TNFα followed by Op. HK-*F. alocis* (TNF + Op.HK-F.a), TNFα followed by UV-*F. alocis* (TNF + Op. UV-F.a). All bacterial conditions were done at multiplicity of infection (MOI) of 10.). (A-B) Data are expressed as the mean ± SEM of the change in 2′,7′-dichlorofluorescein fluorescence per min. (C-D-E) Data are expressed as the mean ± SEM as area under the curve (A.U.C.) between 0-180 min of intracellular ROS production. One way ANOVA and Tukey posttest, n=3. * $p<0.05$; ns=non-significant.

Next, the kinetic assay was used to test the ROS production induced by *F. alocis* when IL-8 was used as a priming agent. Similar to TNFα priming, viable *F. alocis* was able to significantly downregulate the respiratory burst response of IL-8 primed cells (Fig 8B-C). Unprimed and IL-8 primed neutrophils showed a similar high icROS production when challenged with heat-killed *F. alocis* (Fig 8B and D). However, in contrast to TNFα priming conditions, UV-killed *F. alocis* elicited minimal icROS production in the presence or absence of IL-8 priming (Fig 8B and E).

Figure 8

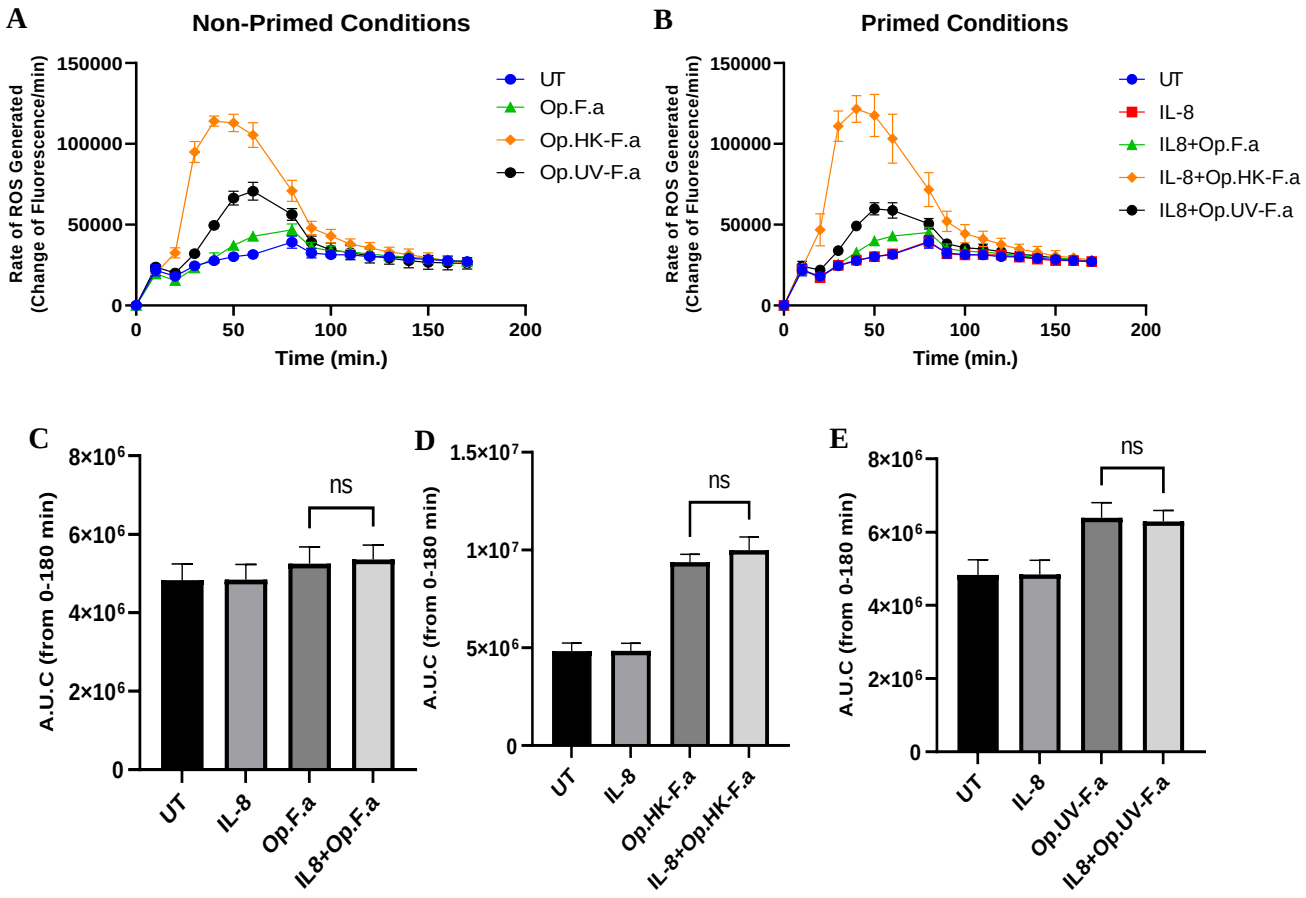
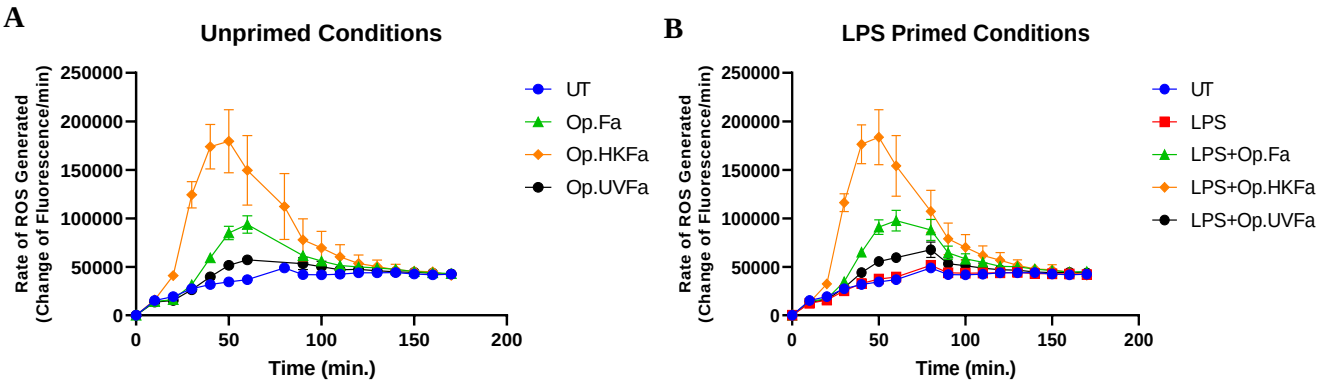


Fig 8. The effect of IL-8 priming on the respiratory burst response induced by F. alocis in human neutrophils.

The intracellular production of reactive oxygen species (icROS) was monitored for up to 180 min. Neutrophils ($1x10^6$ cells/mL) were untreated (UT), incubated with IL-8 (10nM; 60 min), challenged with serum opsonized *F. alocis* (Op. F.a, ATCC 38596), serum opsonized heat-killed *F. alocis* (Op.HK-F. a), serum opsonized UV-killed *F. alocis* (UV-*F. alocis*), or IL-8 followed by Op *F. alocis* (IL-8 + Op. F.a), IL-8 followed by Op. HK-*F. alocis* (IL-8 + Op.HK-F.a), IL-8 followed by UV-*F. alocis* (IL-8 + Op. UV-F.a). All bacterial conditions were done at an MOI of 10. (A-B) Data are expressed as the mean ± SEM of the change in 2′,7′-dichlorofluorescein fluorescence per min; (c-e) quantification of intracellular ROS production as the area under the curve (A.U.C.) between 0-180 min. One way ANOVA and Tukey posttest, n=3. ns=non-significant.

Next, I tested the icROS production induced by *F. alocis* in LPS-primed neutrophils. Priming by *E. coli* LPS, signaling through TLR2 and TLR4 due to contaminated lipoproteins, ultra-purified LPS, signaling only through TLR4, and *P. gingivalis* LPS, which does not activate TLR4 signaling, were tested. Similar to the results obtained with the two other priming agents, *F. alocis* induced minimal icROS production and modulated the LPS-priming response independently of which LPS priming agent was used (Fig 9B and C). The icROS production elicited by heat-killed-*F. alocis* (Fig 9C and F) and UV-killed *F. alocis* (Fig 9D and E) were similar in the presence or absence of LPS priming. A minimal but significant increase in icROS production was observed by UV-killed *F. alocis* when neutrophils were primed with the *E. coli* LPS that can activate TLR2 (Fig 9E). However, the responses elicited by the other LPS priming agents were non-significant. The purified *E. coli* LPS and *P. gingivalis* LPS priming conditions do, however, have similar mean icROS production values; however, this is just an observed trend with no statistical significance. Additional biological replicates will need to be performed to yield a significant increase possibly.

Figure 9



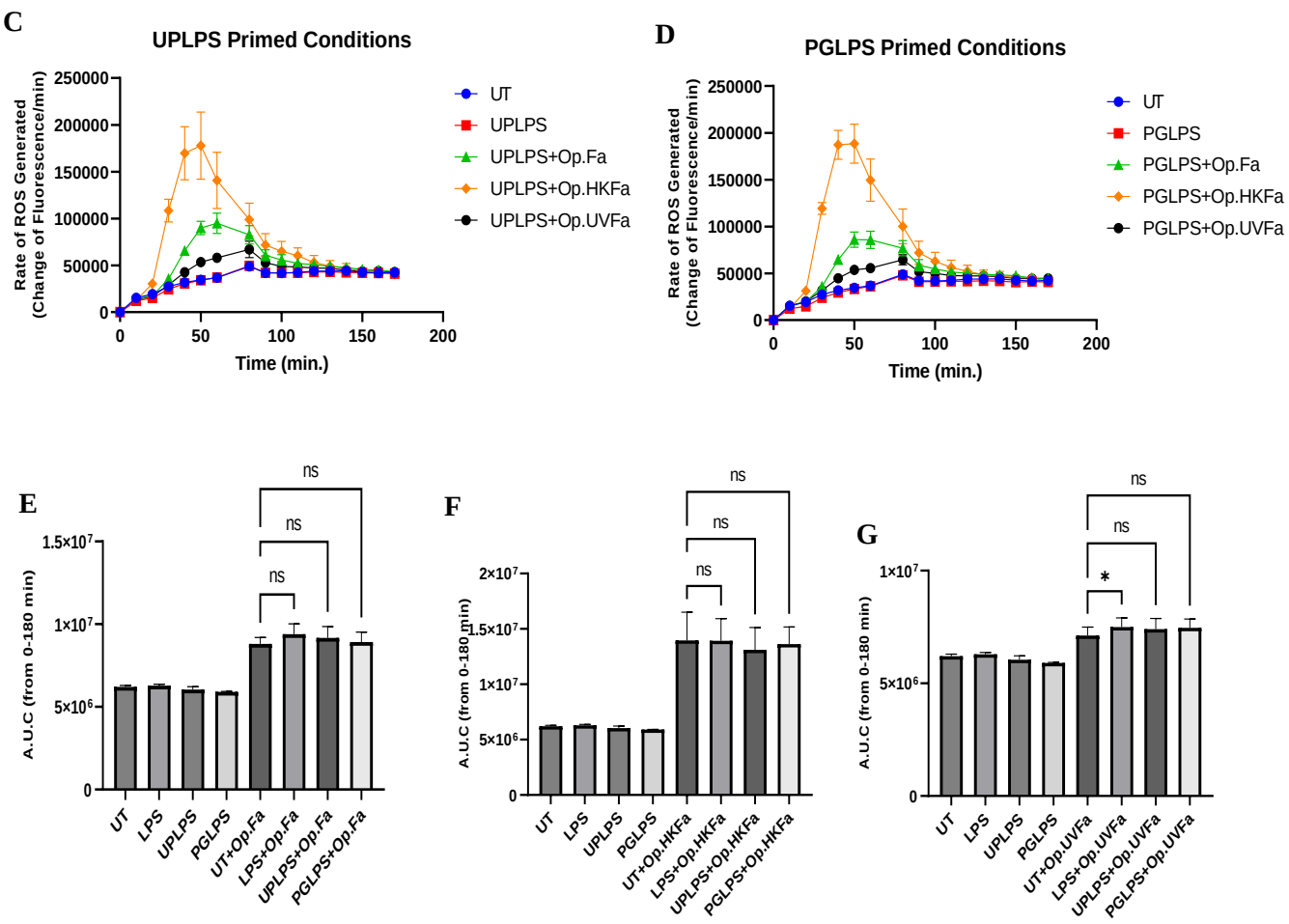


Fig 9. The effect of three different LPS priming conditions on the respiratory burst response induced by F. alocis in human neutrophils. The intracellular production of reactive oxygen species (icROS) was monitored for up to 180 min. Neutrophils (1x10^6 cells/mL) were untreated (UT), incubated with *E. coli* LPS (LPS), ultrapure *E. coli* LPS (UPLPS), or *P. gingivalis* LPS (PGLPS) (10ng/mL; 60 min), challenged with serum opsonized *F. alocis* (Op. F.a, ATCC 38596), serum opsonized heat-killed *F. alocis* (Op.HK-F. a), serum opsonized UV-killed *F. alocis* (UV-*F. alocis*), or LPS treatment followed by Op *F. alocis* (LPS + Op. F.a, UPLPS + Op. F.a, and PGLPS + Op. F.a), LPS followed by Op. HK-*F. alocis* (UPLPS + Op.HK-F.a, UPLPS + Op.HK-F.a, and PGLPS + Op.HK-F.a), LPS followed by UV-*F. alocis* (LPS + Op. UV-F.a, UPLPS + Op. UV-F.a, and PGLPS + Op. UV-F.a). All bacterial conditions were done at an MOI of 10. (a-d) Data are expressed as the mean ± SEM of the change in 2′,7′-dichlorofluorescein fluorescence per min; (e-g) quantification of intracellular ROS production as the area under the curve (A.U.C.) between 0-180 min. One way ANOVA and Tukey posttest, n=3. * $p<0.05$; ns=non-significant.

Overall, the data I generated measuring the icROS production suggest that *F. alocis* modulated the neutrophil respiratory burst response independently of how the cells were primed. Furthermore, the results suggest that the ability of *F. alocis* to modulate the neutrophil response might be associated with a bacterial surface component; however, more specific experiments investigating this phenotype and its cause will need to be performed before conclusions can be drawn.

F. alocis survival is independent of neutrophil priming by TNFα.

Upon bacterial internalization, neutrophils quickly mount antimicrobial responses such as the respiratory burst response and granule fusion to the phagosome to promptly kill the pathogen. However, previous studies have shown that *F. alcois* can survive in neutrophil phagosomes for up to 20 hours post-challenge [4]. Therefore, I next investigated whether TNFα priming would improve the ability of neutrophils to kill *F. alocis*. Bacterial survival was quantified by colony-forming units (CFU) of intracellular and extracellular bacteria obtained from unprimed and TNFα-primed neutrophils after one hour of *F. alocis* challenge. Preliminary data show a similar percentage of intracellular (Fig 10A) and extracellular (Fig 10B) *F. alocis* survival by unprimed and TNFα primed neutrophils. The data suggest a higher percentage of extracellular bacterial survival than intracellular bacteria. The results suggest that the ability of *F. alocis* to survive extracellularly is stronger than once internalized. Furthermore, once internalized, *F. alocis* intracellular survival was independent of TNFα priming. However, this data only represents one trial, and more biological replicates will need to be performed before conclusions can be drawn.

Figure 10

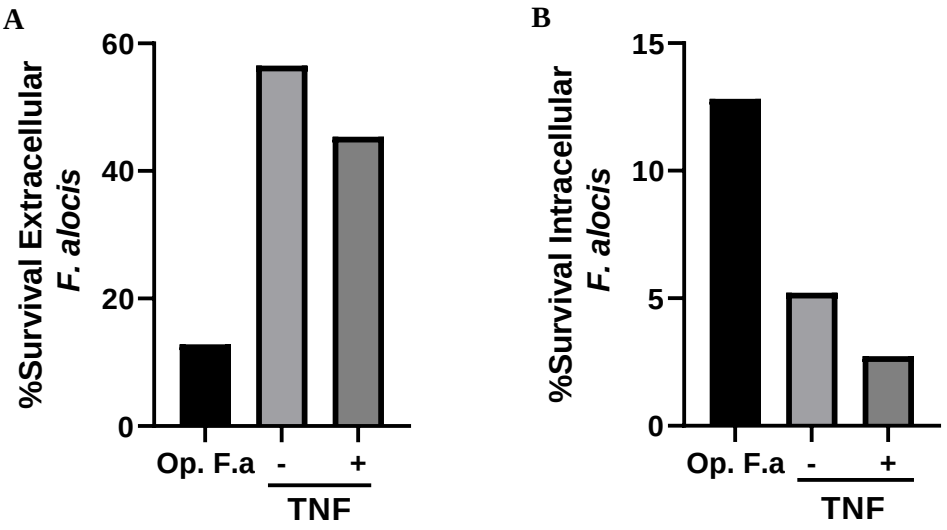


Fig 10. The effect of TNF*α* priming on neutrophil killing of *F. alocis*. Neutrophils (4 x 10^6 cells/mL) were unprimed (TNF-) or primed with TNFα (2ng/ml; 10 min) (TNF+) and challenged with opsonized *F. alocis* at an MOI of 10. Time points were collected at 0 min and 60 min. Bacteria alone (Op. F.a), extracellular bacteria (10A), and intracellular bacteria (10B) were quantified by diluting, plating, and culturing the surviving *F. alocis* post-challenge. Counting colony forming units was performed by eye, and viable colony counts were multiplied by their respective dilution and plating factors. Data expressed as a percentage of *F. alocis* survival by dividing 60 min CFU/mL by 0 min CFU/mL. n=1.

Discussion

Periodontitis is a dysbiotic inflammatory disease promoted by disrupting host-microbe homeostasis leading to non-resolving inflammation and tissue destruction. As a result, circulating neutrophils and those that arrive at the periodontal pocket are exposed to inflammatory mediators such as TNFα, IL-8, and LPS. The inflammatory environment empowers neutrophils with enhanced capabilities to respond upon encountering a stimulant. This new state of hyperresponsiveness is known as priming. Primed neutrophils are present in the gingival tissue,

and their functional response toward periodontal pathogens, like *F. alocis*, is currently unknown. This project aimed to represent the *in vivo* environment by performing *in vitro* priming experiments and defining neutrophil effector functions against *F. alocis*. The results obtained from this study showed enhanced phagocytosis of *F. alocis* by TNFα and LPS-primed neutrophils. However, the increased bacterial internalization did not result in augmented ROS production as expected. Furthermore, preliminary results indicate that *F. alocis* survival was similar in unprimed and TNFα-primed neutrophils. These results suggest that *F. alocis* can modulate the killing capacity of primed neutrophils.

Different receptors mediate priming by endogenous and exogenous priming agents on immune cells. For example, TNFα, IL-8, and LPS are all known priming agents recognized via different receptors, TNFR1/TNFR2, CXCR1/CXCR2, and TLR4 receptors, respectively [26-28]. Activation of different receptors by the diverse agonists involved in priming induces similar molecular events required for priming. For example, activation of p38 MAPK signaling contributes to TNF priming, intracellular calcium mobilization, ERK, and PI3K signaling are associated with IL-8 priming, and IRAK-4 and the MAPK signaling are involved in LPS priming [19, 29]. Our data showed that in contrast to TNFα and LPS-priming, IL-8-primed neutrophils had no enhanced phagocytosis when infected with *F. alocis*. Additional experiments are required to determine how *F. alocis* dismantled IL-8 signaling pathways to prevent priming.

Bacterial-derived products like LPS and lipopeptides are abundant in the gingival crevice. Neutrophil priming by LPS relies on TLR4 recognition and the activation of downstream signaling pathways. Our data showed that *E. coli* LPS primed neutrophils with and without lipoprotein contamination for enhanced phagocytosis towards *F. alocis*. However, exposure of neutrophils to *P. gingivalis* LPS did not prime them for enhanced phagocytosis. One of *P. gingivalis* evasion

strategies relates to modifications of its LPS to either antagonize or be inert to TLR4 recognition. Lipid A, a major structural component of LPS, is typically phosphorylated in Gram-negative bacteria; however, the LPS of *P. gingivalis* is non-phosphorylated, causing a significant structural change. This modification in *P. gingivalis* lipid A structure makes LPS inert for TLR4 activation [30]. This modification may be a strategy of *P. gingivalis* to avoid immune surveillance. By avoiding recognition by TLR4, *P. gingivalis* fails to prime neutrophils, thus leading to an unprimed response upon a bacterial challenge that would be advantageous to the pathogen. As a keystone pathogen of periodontitis, avoiding immune surveillance and invoking a non-primed response from immune cells would allow for *P. gingivalis* to persist in the oral biofilm allowing for the modification of microbial community to make it more suitable for the colonization of other periodontal pathogens, such as *F. alocis* [8].

Chronic inflammation is one of the hallmarks of periodontitis and has been observed experimentally *in vivo,* eventually leading to alveolar bone loss and tooth loss [31]. The constant high levels of cytokines, chemokines, and bacteria-derived products present during chronic inflammation primes neutrophils. *F. alocis* is found in high abundance in periodontitis sites, which led me to hypothesize that this periodontal pathogen could modulate the antimicrobial responses of primed human neutrophils. The *in vitro* priming experiments, independently of which priming agent was used, showed low icROS generation by neutrophils when challenged with live *F. alocis* to levels comparable to the unprimed response. These results allow me to speculate that the ability of *F. alocis* to manipulate ROS generation by both primed and unprimed neutrophils may be how this organism contributes to prolonging inflammation within the periodontium. Upon phagocytosis of particulate stimuli, like a bacterium, neutrophils mount a high ROS production, accelerating their apoptotic program. Macrophage clearance of apoptotic neutrophils through efferocytosis

triggers the resolution of inflammation [32]. Additionally, the Uriarte lab recently showed that *F. alocis* prolongs the lifespan of neutrophils [33]. Therefore, reducing the amount of icROS generated may be a strategy by *F. alocis* to promote its survival, delaying neutrophil apoptosis and contributing to exacerbating inflammation.

An interesting phenotype observed by killed *F. alcois* was that the pathogen lost the ability to modulate the primed icROS response of neutrophils. These results suggest a possible virulence factor lost upon artificially killing treatment, rendering *F. alocis* unable to modulate the primed icROS response. UV-killing of bacteria results in the inability to secrete metabolites, but surface structures on the bacteria remain intact. On the other hand, the heat-killing of bacteria denatures surface structures but can make the bacterium leakier of inactive metabolites. The heat-killing and UV-killing of *F. alocis* caused an enhanced icROS response in TNFα-primed neutrophils. However, heat-killing *F. alocis* significantly increased icROS production in unprimed and primed cells compared to live and UV-killed bacterium. However, UV-killing-*F. alocis* lost the ability to modulate ROS production by TNFα-primed neutrophils. These findings suggest that there may be two virulence factors at play. One is an active metabolite that modulates the primed responses of neutrophils lost upon UV- and heat-killing. The other virulence factor might be a thermosensitive bacterial surface component responsible for downregulating the icROS response. The picture is muddled, however, by the fact that in some cases with other priming agents such as IL-8 and purified *E. coli* LPS, there is no observed enhancement of the icROS response upon challenge by UV-killed *F. alocis*. Many possibilities may be at play here, such as a surface component being able to modulate some priming agent signals but not others or perhaps further biological replicates need to be performed to show significant differences between primed and unprimed responses towards UV-killed *F. alocis*. Nevertheless, further experiments specifically

designed to address these questions will need to be performed before conclusions can be drawn about these hypothetical virulence factors.

As professional phagocytes, the phagocytosis of pathogens by neutrophils is essential to their ability to control infection. Upon phagocytosis, neutrophil granules fuse with the bacteria-containing phagosome, releasing their antimicrobial peptides and NADPH oxidase complex components to create a hostile and toxic environment to kill the internalized pathogen [34]. The data generated in this project shows that TNFα and LPS, but not IL-8, primed neutrophils had an enhanced phagocytic response towards *F. alocis*. Since each priming agent tested in this project bind to different receptors, it is possible to speculate that *F. alocis* can modulate IL-8 signaling, but not TNFα or TLR4, to prevent enhanced phagocytosis. An intermediate component within the IL-8-mediated signaling cascade may be susceptible to *F. alocis* modulation. Because the IL-8-primed respiratory burst response appears modulated by *F. alocis*, it suggests that bacteria internalization might not be necessary to modulate the respiratory burst response, consistent with previous reports that *F. alocis* can modulate neutrophil function from a distance [35]. In the case of TNFα and LPS priming, however, *F. alocis* can still modulate the respiratory burst response when internalized. The redirection and delay of neutrophil granule recruitment to the *F. alcois*-containing phagosome may achieve this response. *F. alocis* has been shown to delay specific granule fusion to the phagosome [17]. Specific granules contain 60% of the membrane-bound components of the NADPH oxidase complex [36]; therefore, its delay would be expected to decrease icROS. However, another neutrophil function enhanced by priming is granule exocytosis and neutrophils' antimicrobial capabilities. The effect of priming on *F. alocis'* ability to delay granule recruitment is still unknown and will be explored in future studies. Additionally, further

investigation needs to be performed to identify the virulence factor(s) attributable to this granule redirection.

Overall, these results indicate that *F. alocis* can modulate the antimicrobial responses of primed human neutrophils. TNFα and *E. coli* LPS, but not IL-8, priming of human neutrophils showed enhanced phagocytosis of *F. alocis*. Furthermore, exposure to *P. gingivalis* LPS failed to prime human neutrophils due to the failure to signal through TLR4. Together, these modulations may increase the survival of *F. alocis in vivo* and sustain the chronic inflammation associated with periodontitis.

Conclusions and Summary

F. alocis modulates the antimicrobial responses of primed human neutrophils to promote its survival and the progression of periodontitis. Even though TNFα and LPS primed neutrophils display enhanced phagocytic ability to internalize *F. alocis*, the oral pathogen can still induce minimal respiratory burst response, similar to the levels induced in unprimed neutrophils. Interestingly, phagocytosis was similar between IL-8 primed and unprimed cells, suggesting that *F. alocis* can modulate the IL-8 signaling pathway to prevent its phagocytosis. Additionally, the atypical LPS of the oral pathogen *P. gingivalis* was unable to prime neutrophils, most likely due to its inability to activate TLR4, an evasion strategy used by this oral bacterium to avoid immune surveillance. Preliminary data also suggest that *F. alocis* survival is independent of TNFα priming of neutrophils. These *in vitro* results demonstrate how *F. alocis* can modulate the antimicrobial responses of primed neutrophils to promote its survival and exacerbate the inflammation that is beneficial to the dysbiotic oral community present in periodontitis in a manner more representative of *in vivo* conditions (Fig 11).

Figure 11

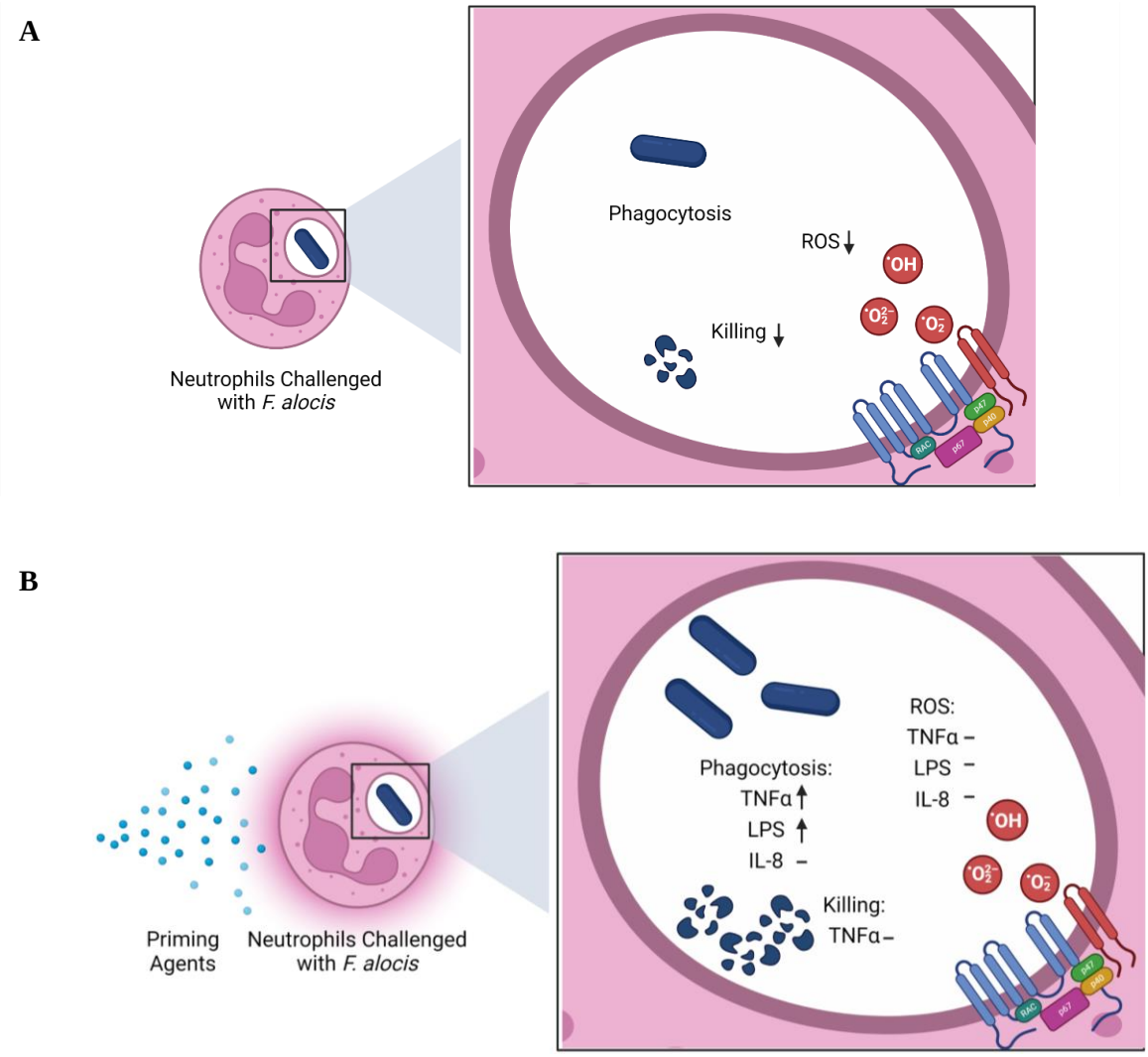


Fig 11. Summary of *F. alocis* modulations of primed human neutrophil antimicrobial responses. (a) Unprimed neutrophils challenged with *F. alocis* display efficient phagocytic ability of *F. aloclis* but reduced ROS production and killing ability. (b) Neutrophils were primed with TNFα, IL-8, or *E. coli* LPS followed by *F. alocis* challenge. Phagocytosis was enhanced in TNFα and *E. coli* LPS-primed neutrophils. In addition, no significant difference in icROS production induced by *F. alocis* was observed between unprimed and primed cells. Preliminary data also suggests no difference in killing ability of *F. alocis* between unprimed neutrophils and neutrophils primed with TNFα.

Future Directions

Other neutrophil antimicrobial responses, such as granule recruitment to phagosomes and exocytosis of granule contents into the extracellular matrix, are also subject to priming. *F. alocis* can delay and prevent specific and azurophilic granule recruitment to the bacteria-containing phagosome [17]; however, it is unknown if *F. alocis* can modulate this response in primed neutrophils. The knowledge gained from these experiments would offer a complete picture of how *F. alocis* modulates the primary antimicrobial responses of primed neutrophils. In addition, viability assays would demonstrate how the modulation of these responses culminates in the overall ability of neutrophils to kill *F. alocis*. Preliminary data I have generated by comparing the survival of *F. alocis* challenged with unprimed and TNFα-primed neutrophils suggest that *F. alocis* can survive in the presence of primed neutrophils just as well as in the presence of unprimed neutrophils. However, more biological replicates are needed before conclusions can be drawn from this result, and the other priming agents from this project also need to be assessed.

Other priming agents not tested in this project are known to prime neutrophil antimicrobial responses. For example, complement component 5a (C5a) and granulocyte-macrophage colony-stimulating factor (GM-CSF) are present during infection [19]. Observing how neutrophils primed with these other priming agents respond to *F. alocis* challenge would further reflect *in vivo* conditions. Additionally, many different cytokines, chemokines, and microbial products are present and available to prime neutrophils during infection. The recruited neutrophils are likely exposed to many different priming agents during their journey to the site of infection. Thus, to further reflect *in vivo* conditions, priming neutrophils with multiple priming agents simultaneously may offer a more representative look into *in vivo* neutrophil-*F. alocis* interactions.

As previously mentioned, an interesting phenotype observed was that *F. alocis* lost its ability to modulate the primed respiratory burst response upon exposure to heat or UV killing of the bacterium. One virulence factor may be an actively secreted metabolite that modulates the primed respiratory burst response, as indicated by the significantly higher icROS amounts produced by primed neutrophils compared to unprimed neutrophils when challenged with UV-killed *F. alocis*. Another possible virulence factor may be a surface structure or molecule lost upon the bacteria's heat-killing, which is responsible for the downregulation of the respiratory burst response. These virulence factors could be further investigated by the isolation of metabolites secreted by *F. alocis* and protease treatment, and isolation of surface components of *F. alocis*. Protein sequencing could then reveal the identity of molecules in these isolates, and those unique to *F. alocis* could be used to stimulate icROS production in neutrophils.

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