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ORIGINAL ARTICLE

Fusobacterium nucleatum dissemination by neutrophils

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ABSTRACT

Recent studies uncovered that *Fusobacterium nucleatum (Fn)*, a common, opportunistic bacterium in the oral cavity, is associated with a growing number of systemic diseases, ranging from colon cancer to Alzheimer's disease. However, the pathological mechanisms responsible for this association are still poorly understood. Here, we leverage recent technological advances to study the interactions between Fn and neutrophils. We show that Fn survives within human neutrophils after phagocytosis. Using in vitro microfluidic devices, we determine that human neutrophils can protect and transport Fn over large distances. Moreover, we validate these observations in vivo by showing that neutrophils disseminate Fn using a zebrafish model. Our data support the emerging hypothesis that bacterial dissemination by neutrophils is a mechanistic link between oral and systemic diseases. Furthermore, our results may ultimately lead to therapeutic approaches that target specific host-bacteria interactions, including the dissemination process.

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Introduction

Epidemiological studies reveal a strong association between periodontal disease and various systemic illnesses and are reinforced by emerging mechanistic links [1]. Translocation of microorganisms from the oral cavity to other organs has been suggested in conditions including gastrointestinal disorders (e.g. colorectal carcinomas and adenomas [2,3], inflammatory bowel disease [4], appendicitis [5]), cardiovascular disorders (e.g. atherosclerosis [6], cerebral aneurysm [7], Lemierre's syndrome [8]), neurological disorders (e.g. Alzheimer's disease [9], reviewed in [10]), and adverse pregnancy outcomes [11–15]). *Fusobacterium nucleatum* (*Fn*), a predominant bacterial species of the oral cavity [16], has been associated with these conditions. *Fn* often serves as a bridging species between early and late colonizers to facilitate the formation of oral biofilms [16]. *Fn* is usually considered a commensal species [17], as it is commonly detected in the oral cavity [2,3,10,16]. A significant gap in our knowledge remains on how *Fn* can disseminate to distant and non-oral sites without being cleared by the immune defense systems.

The traditional paradigm for oral pathogen dissemination is that oral bacteria travel through the bloodstream and the lymphatic system [18]. However, *Fn* has not been reproducibly recovered from the blood of patients with periodontitis, minimizing the potential role of *Fn* bacteremia as a mechanism. An alternative hypothesis emerges, revolving around the role of innate immune cells in transporting pathogens and dispersing them. This hypothesis, also known as the Trojan Horse hypothesis [19], is supported by examples from other immune cells and pathogens. For example, macrophages contribute to HIV dissemination [20,21] and transport *Chlamydia* [22]. Monocytes have been reported to transport visna viruses [23], *Listeria monocytegenes*, and *Cryptococcus neoformans* to the mouse brain [24–28]. Dendritic cells can transport HIV exosomes [29]. Myeloid cells are a reservoir for Zika virus replication [30], and microglia can disseminate the virus from the mother to the fetal brain [31]. Dendritic cells disseminate *Toxoplasma gondii* to the brain and other parts of the body [32,33]. In the context of periodontal diseases, dendritic cells were shown to transport *Porphyromonas gingivalis* from the oral cavity to the arterial walls [34]. Neutrophils were demonstrated to transport *Staphylococcus aureus* [35,36] and *Bacillus anthracis* [37]. Although there are several possible mechanisms on how innate immune cells could help pathogens to

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be transported within the circulation without getting recognized by the other immune checkpoints, the exact mechanisms remain unknown.

Recent studies have shown that *Fn ss nucleatum* (*Fnn*) and *Fn ss polymorphum* (*Fnp*) suppress the oxidative burst in a neutrophil-like cell line after phagocytosis [38]. The effect was subspecies-specific, with higher levels of neutrophil cell death for *Fnp* than for *Fnn*. However, a significant proportion of neutrophils remained viable following interaction with *Fnn*. Together, low antibacterial responses and sustained neutrophil viability support a model in which host neutrophils serve as an intracellular niche for *Fnn* survival and mediate their dissemination throughout the body.

Here, we test the hypothesis that neutrophils can disseminate viable, intracellular *Fnn*. We quantify the dissemination of *Fn* by primary human neutrophils using *in vitro* microfluidic devices. We validate these observations *in vivo* by showing that neutrophils disseminate *Fn* using a zebrafish model. To investigate whether bacterial surface composition modulates interaction with neutrophils, we compare the behavior of neutrophils laden with wild-type *Fnn* versus *Fnn Fap2* and *RadD* mutants, both *in vitro* using primary human neutrophils and in our *in vivo* zebrafish model.

Methods

Bacterial strains and inoculum preparation

Fnn (ATCC 25,586), *Fnp* (ATCC 10,953) and *Streptococcus mitis* ATCC 49,456 were maintained on blood agar plates and in brain-heart infusion broth. Bacteria were anaerobically grown under 5% CO_2, 10% H_2–85% N_2 atmosphere at 37°C for 4–6 days. Bacterial suspensions were prepared from primary cultures at their log phase of growth, and concentrations were determined by spectrophotometry corresponding to 1×10^9 bacteria/ml. *S. mitis* was used as a non-*Fn* and an oral bacterium control. Fap2 and RadD gene-inactivated adhesion mutants were a kind gift from Professor Wenyuan Shi [39].

Human neutrophil isolation, phagocytosis of Fn by neutrophils, and viability of intracellular Fn after neutrophil phagocytosis

The biological sampling protocol was approved by the Forsyth Institute's Institutional Review Board (Pr. No: 17-03). All subjects provided written consent before blood donation. Neutrophils were obtained from medically and periodontally healthy donors (male and female) between 18–35 years old. Fifty ml of peripheral venous blood was collected into heparinized tubes.

Neutrophils were isolated using a discontinuous gradient system, as previously reported [40]. Contaminating erythrocytes were lysed with isotonic NH_4Cl buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 120 mM EDTA; pH 7.4); 90–95% pure neutrophils were obtained as determined by Giemsa stain and light microscopy. Neutrophil viability was greater than 95%, as determined by trypan blue exclusion. After 24 hours of anaerobic growth in brain-heart infusion broth, supplemented with hemin in an anaerobic chamber with 85% N_2, 5% H_2, and 10% CO_2, *Fn* were harvested by centrifugation; washed three times with sterile, pyrogen-free saline; incubated; and labeled with fluorescein isothiocyanate (FITC; 100 µg/ml of PBS) [41]. Six different conditions were tested: 1) neutrophils were cultured alone (in broth). 2) neutrophils were cultured with *Fnp*. 3) neutrophils were cultured with *Fnn*, 4) neutrophils were cultured with *Fnn* with *Fap2* deletion, and 5) neutrophils were cultured with *Fnn* with *RadD* deletion. 6) neutrophils were cultured with *S. mitis*.

We calculated the phagocytic index for purified neutrophils based on the percentage of neutrophils containing bacteria and the number of bacteria within each neutrophil at different MOIs of 1, 20, and 100. We estimated phagocytosis using imaging flow cytometry (AMNIS ImageStream). We identified neutrophils by separating nucleated cells (Hoechst or Draq5+) based on side scatter. To confirm the viability of intracellular bacteria, we treated *Fn*-laden neutrophils with gentamicin, a non-cell permeable antibiotic that kills extracellular but not intracellular bacteria [42]. This approach restricted our observations to neutrophil-passaged bacteria. We incubated bacteria with neutrophils at different MOIs for 30 mins. Then, the cells were treated with gentamicin for 1 hour, washed out of the drug, and incubated for a further 3.5 hours. At 5 hours post-infection, we centrifuged samples. We lysed the neutrophils on ice with gentle sonication to disrupt the cell membrane. We then plated out the supernatant and the lysed neutrophil pellet on blood agar media to compare the viability of extracellular bacteria in the supernatant to intracellular bacteria in the neutrophil pellet. Intracellular bacteria viability was measured after 4 days of plating and expressed as square root transformation of colony-forming units (CFU). As a control to verify gentamicin effectiveness, we used gentamicin-untreated cells and demonstrated that the numbers of bacteria that grew were higher including the surface-attached *Fn* as a control (data not shown).

Human neutrophil dissemination of Fn in microfluidic devices

Human neutrophils were isolated from fresh peripheral blood samples from healthy donors using negative selection (EasySep. Human Neutrophil Isolation Kit, Cat# 17957, StemCell). We stained

the neutrophil nuclei with 15 µM Hoechst dye 33,342 (Sigma Aldrich) to allow automated image analysis. We assessed *Fn* phagocytosis by neutrophils after labeling *Fn* with AlexaFluor-488 as previously described [43,44]. We confirmed phagocytosis by co-labeling bacteria with the fluorescent pH sensor pHrodo, which increases intensity as the phagosome is acidified [43]. Labelled bacteria were loaded into microfluidic devices at a concentration of 1×10^7 cells/mL, then neutrophils were introduced at a concentration of 1×10^7 cells/mL. Comparisons to unladen neutrophil were made by loading with 100 nM of fMLP in place of bacteria. Neutrophils laden with intracellular bacteria in microfluidic mazes were identified based on Hoechst and pHrodo fluorescence positivity and tracked using the FIJI manual tracking plugin (Fiji Is Just Image J, NIH) as previously described [45].

Zebrafish model of local Fn dissemination

We used existing transgenic zebrafish strains in which neutrophils express GFP driven by the *myeloperoxidase* promoter [46,47]. Bacteria were stained using AlexaFluor-594 (red-fluorescent dye). To measure dissemination, we microinjected bacteria into the developing otic vesicle (developing ear), which provides a useful compartment from which to observe dissemination [48,49]. Following microinjection, larvae in which bacterial delivery was successfully restricted to the otic vesicle were selected for live imaging studies.

We compared dissemination by neutrophils by counting the number of bacteria-laden cells of each type observed to exit the site of infection. Neutrophils laden with bacteria in zebrafish were identified based on coincidence of neutrophil GFP and AlexaFluor-594 positivity of bacteria, and positions collected manually in FIJI from collapsed z-stacks. Distribution was calculated as the average distance of laden neutrophils from the geometric centroid (average X and Y of the distribution). To test the ability of bacteria to disseminate in the absence of leukocytes, we suppressed leukocyte specification by microinjecting antisense morpholino oligonucleotides targeting *spi1* and *csf3r* into zebrafish embryos at the 1-cell stage of development and raising to the larval stage before infection, as previously described in detail [50]. For morpholino experiments where neutrophils were absent, the XY position of each fluorescent bacterial signal was identified based on thresholding and particle analysis, with distribution distance calculated relative to the largest fluorescent object, which represented the infection bolus.

Statistics

All data are presented as the average of at least three experiments repeated in triplicate. For comparison between the two groups, Student's t-test was applied for significance. For multiple group analysis, we applied one-way ANOVA with post hoc Bonferroni or least significance difference (LSD) corrections, where appropriate. For between-group data comparisons, we used a non-parametric Kruskal-Wallis test followed by a post-hoc test in cases of statistical significance. Between-group comparisons were accomplished by ANOVA; 95% confidence levels were calculated.

Results

Fn *survives within human neutrophils*

We measured the survival of *Fnn* and *Fnp* in primary peripheral blood neutrophils from healthy donors (Figure 1). We compared the survival of *Fnn* and *Fnp* with that of *Fnn* with the deletion of the *Fap2* and *RadD* genes. We used *S. mitis*, a human oral species killed by neutrophils after phagocytosis, as a control. Gentamicin was used to eliminate any bacteria that were not phagocytosed during these experiments. *S. mitis* did not show any survival in neutrophils at any MOI tested. *Fnp* showed a dose-dependent survival in neutrophils, with the highest survival at MOI 100. Meanwhile, *Fnn* survival was similar at 20 and 100 MOI suggesting a lower infectivity of *Fn* at lower MOI. The deletion of *Fap2* gene did not affect the survival of *Fn* in human neutrophils. Interestingly, *RadD* deletion enhanced the survival of *Fn* in human neutrophils even at the lowest MOI (MOI = 1) tested compared to the wild-type *Fn* strain ($p < 0.05$).

Fn is disseminated by primary human neutrophils *in vitro*

We found that bacteria-laden neutrophils remain motile following phagocytosis. To measure neutrophil motility post-phagocytosis, we designed grid-pattern microfluidic devices. We loaded these devices first with bacteria and then with primary human neutrophils. Host-pathogen interactions were directly monitored using timelapse photography. We then measured the velocity of neutrophils laden with *Fnn*, *Fnp*, and *S. mitis* (Figure 2). We observed that neutrophils continued moving after phagocytosing *Fn* (Figure 2). Neutrophil average velocity after phagocytosis of *Fnn* and *Fnp* was 22.2 ± 13.7 µm and 13.8 ± 11.0 µm, respectively, compared to 17.0 ± 4.4 µm/min after phagocytosis of *S. mitis*.

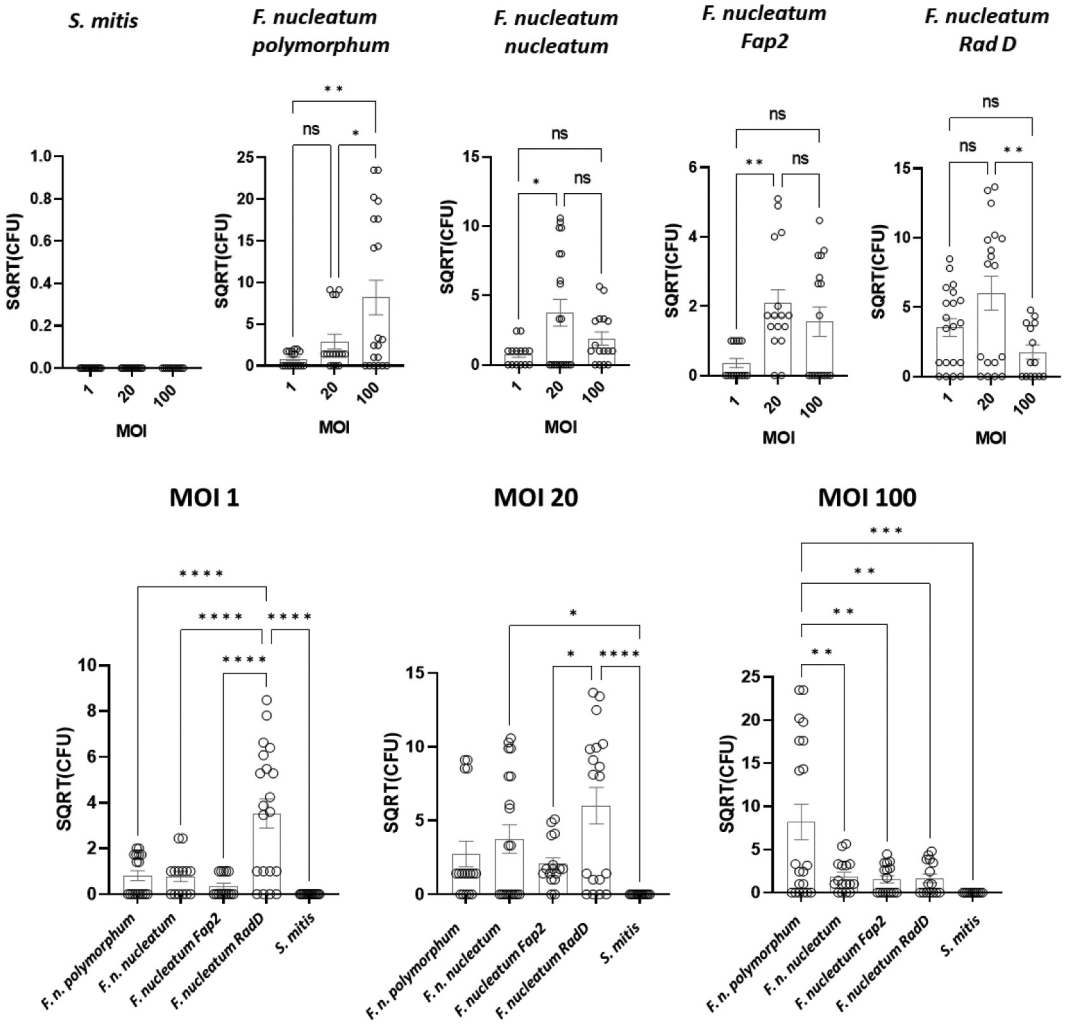

Figure 1. *Fn* survives inside human neutrophils. Human neutrophils from healthy donors were exposed to *Fnn*, *Fnp*, and *Fn* with deletion of the Fap2 or RadD genes at MOI of 1, 20, or 100. After exposure, the neutrophils were washed to remove all *Fn* that was not phagocytosed. To confirm the viability of intracellular bacteria, we treated *Fn*-laden neutrophils with gentamicin for 1 hour, washed them out of the drug, and incubated them for a further 3.5 hours. At 5 hours post-infection, we centrifuged samples and plated out the supernatant and the lysed neutrophil pellet on blood agar media to compare the viability of escaped (extracellular) bacteria in the supernatant to intracellular bacteria in the neutrophil pellet. Intracellular bacteria viability was measured four days after plating and expressed as square root transformation of colony-forming units (CFU). S. mitis, readily killed by neutrophils, was used as a control ($N > 14$). Plots show individual data points, mean and standard error of the mean (SEM).

Zebrafish neutrophils disseminate Fn in vivo

To demonstrate that this biology was not limited to *in vitro* interactions, we tested whether neutrophils could disperse bacteria *in vivo*. We utilized a zebrafish infection model with GFP-expressing neutrophils, where *Fnn*, *Fnp* or *S. mitis* were labeled with AlexaFluor-594 and injected into the otic vesicle at 54 hours post-fertilization (hpf). Timelapse imaging revealed neutrophil recruitment to the site of infection, phagocytosis of bacteria, and dispersal of bacteria away from the infection bolus. Scoring the distribution of neutrophils laden with bacteria at 5 hours post-interaction (hpi) demonstrated that neutrophils appeared to distribute bacteria away from the site of infection following the initial inflammatory response (Figure 3(a)). At 5 hpi, *Fnn* were displaced 76.5 ± 22.5 µm, *Fnp* 77.0 ± 31.25 µm, and *S. mitis* 69.7 ± 25.6 µm (Figure 3(b)). This indicated that neutrophil carriage of intracellular bacteria was relevant to *in vivo* infections and not an artifact of our *in vitro* model.

In vivo Fn dissemination is dependent on leukocytes

To confirm the importance of leukocytes in bacterial dispersal in zebrafish, we suppressed leukocyte differentiation by morpholino (MO) knock-down of the pro-myelopoietic factors *spi1* and *csf3r* at 0 hpf (1-cell stage) as we recently described [48]. We infected the larvae at 54 hpf and measured bacterial

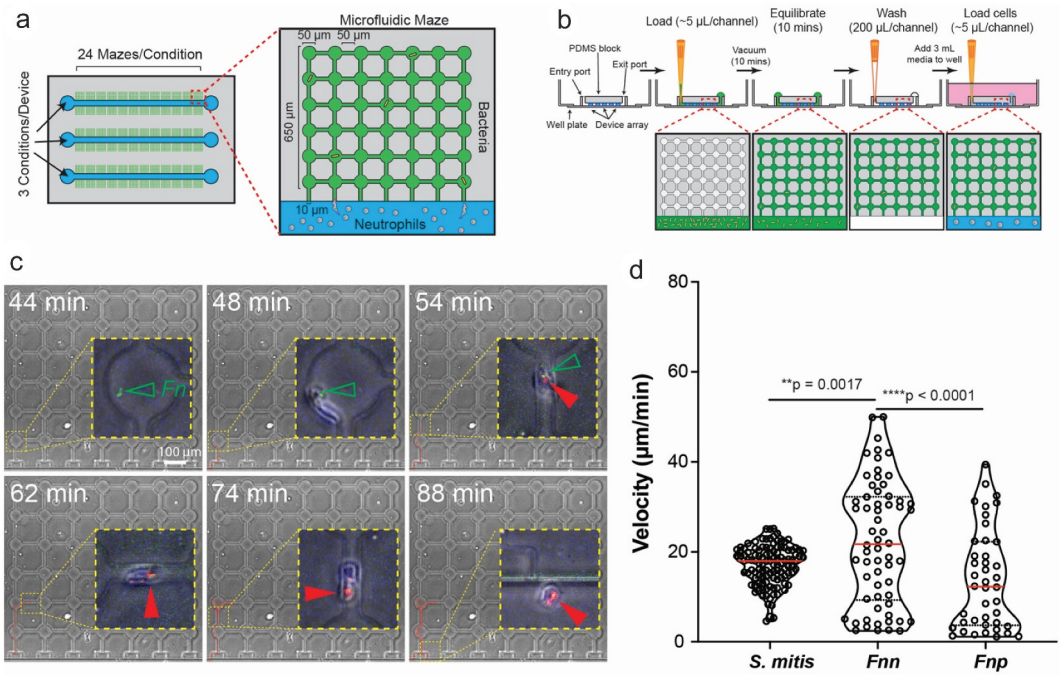

Figure 2. Human neutrophils phagocytose and transport bacteria through microfluidic mazes. a) Diagram detailing the microfluidic device used to measure the migration of neutrophils laden with phagocytosed bacteria. Each device has three independent cell loading channels, and each channel has 24 migration mazes. Each maze (magnified view) fits in one field of view for faster imaging. b) Experimental protocol used. First, bacteria were loaded into the channel, then drawn into the mazes by placing the device under a vacuum. The loading channel was washed with media, leaving the bacteria only in the mazes and the devices submerged in media. Finally, neutrophils were added to the channel. The device was transferred to the microscope stage for time-lapse imaging. The interactions between the moving neutrophils and live bacteria were usually monitored for up to 8 hours. c) Micrographs from a representative time-lapse series showing a neutrophil entering the maze (48 mins), phagocytosing the bacteria, which is acidified (red pHrodo signal 54 mins), and transporting the bacteria around and out of the maze (88 mins). Green and red arrows point at free bacteria and phagocytosed bacteria, respectively. d) Graphs show the velocity of neutrophils migrating in mazes laden with *S. mitis*, *Fnn*, and *Fnp*. Neutrophils laden with *Fnn* exhibited significantly higher velocity than the other species. Statistics: One-way ANOVA with Tukey's multiple comparisons test. Each point is the average velocity of one neutrophil track. Data are pooled from $N = 3$ experiments per condition, $N = 50$ or more neutrophils per condition. Red horizontal line represents the median and the dashed lines represent the first and third quartile.

dispersal at 5 hpi. Because morpholino treatment suppressed leukocyte (neutrophil and macrophage) development, we measured the dispersal of all visible bacteria in this experiment rather than limiting our measurements to bacteria within neutrophils. Dispersal was reduced by 65.4% for *Fnn* ($^{*}p = 0.0137$), 34.8% for *Fnp* ($p = 0.1642$) and 34.6% for *S. mitis* ($p = 0.1487$), in *spi1/csf3r* morphants compared to Control-MO injected larvae (Figure 4). These data show that without leukocytes carrying bacteria away from the infection bolus, *in vivo* dispersal is significantly reduced.

Fn interaction with neutrophils is modulated by bacterial surface composition

Dissemination of bacteria by neutrophils requires both the survival of intracellular bacteria and the maintenance of neutrophil motility. Since *Fnn* survival within neutrophils appeared to be modulated by the presence/absence of bacterial surface adhesins (Figure 1), we asked whether these proteins might also modulate post-phagocytic neutrophil motility. Using our microfluidic assays for primary human neutrophils, we observed that the velocity of neutrophils loaded with *Fap2* (24.98 ± 10.9 µm/min) and *RadD* (27.0 ± 9.5 µm/min) mutants was significantly higher than cells loaded with wild-type *Fnn* (20.4 ± 9.8 µm/min) ($^{**}p = 0.0032$ and $^{****}p < 0.0001$ respectively, Figure 5(a)). The average velocity of neutrophils laden with bacteria of any genotype remained significantly lower than unladen cells migrating in response to fMLP (32.0 ± 13.3 µm/min).

Finally, we tested whether *Fap2* and *RadD* modulated the behavior of bacterially laden neutrophils *in vivo*. Consistent with *in vitro* observations, zebrafish neutrophils laden with *Fnn Fap2* (134.5 ± 87.3 µm, $^{*}p = 0.0473$) and *RadD* mutants (123.2 ± 35.3 µm, $p = 0.1198$) were more broadly distributed at 5 hpi than neutrophils laden with wild-type bacteria (µm/min 85.1 ± 41.8 µm) (Figure 5). These observations demonstrate that bacterial cell surface composition modulates host cell behavior and leukocyte-mediated bacterial dissemination both *in vitro* and *in vivo*.

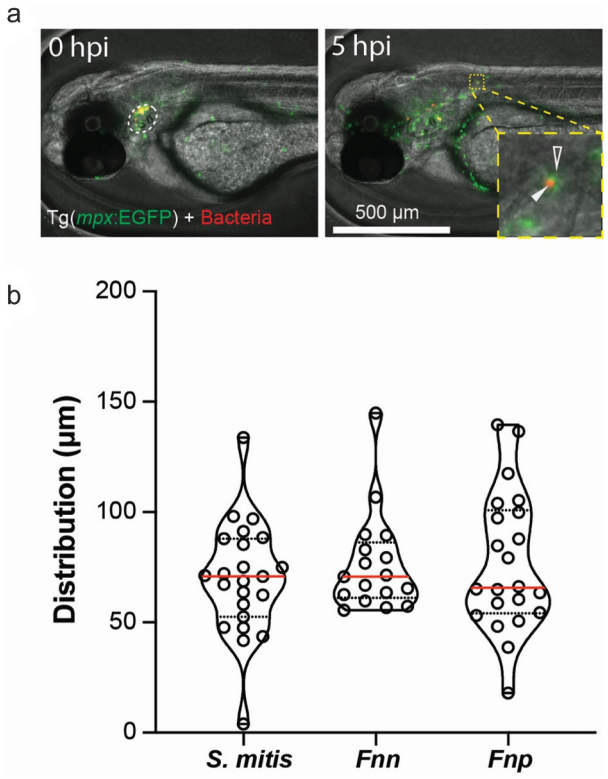

Figure 3.Local dissemination of bacteria in zebrafish. a) Representative images show that host neutrophils (green fluorescence, GFP) disseminate *Fnn* (red fluorescence, AlexaFluor-594) locally. b) Graph shows the distance of local dissemination over the first five hours post-infection is not dependent on *Fn* species. Each point on the graph represents the average distribution of all bacteria-laden neutrophils within a single fish. Red horizontal line represents the median and the dashed lines represent the first and third quartile. Statistics: One-way ANOVA with Tukey's multiple comparisons test.

Discussion

Here, we show that neutrophils disseminate *Fn in vivo* and *in vitro*. We used microfluidic devices and zebrafish infection models for studying host-pathogen interactions. Microfluidic devices enabled detailed and precise measurements of neutrophil interactions with various bacteria [43,51–53]. Strengths of this model include the single-cell resolution of the studies and the ability to scale up and screen dozens of conditions at once in precisely controlled conditions [43,51–53]. Zebrafish larva infection models have been used extensively to characterize leukocyte-mediated dissemination of bacterial and fungal pathogens *in vivo* [54–56]. Strengths of this model include the ability to modulate leukocyte populations prior to infection [50,53] and the capacity for live imaging of transgenically-labeled host cells [47,57] interacting with fluorescently labeled bacteria. Our data validate the utility of these tools in exploring the mechanisms by which neutrophils could disseminate specific bacteria throughout the body.

Previous work from our group demonstrated that primary human neutrophils phagocytosed *Fn* subspecies, which led to the killing of neutrophils through a non-phlogistic and apoptotic mechanism [38]. Through this interaction, neutrophil-mediated oxidative killing was also suppressed in cells cultured with *Fn*, suggesting a mechanism through which the bacteria could evade the immune defense. In addition, the mechanism was likely subspecies-specific. In this study, we confirmed this observation using the

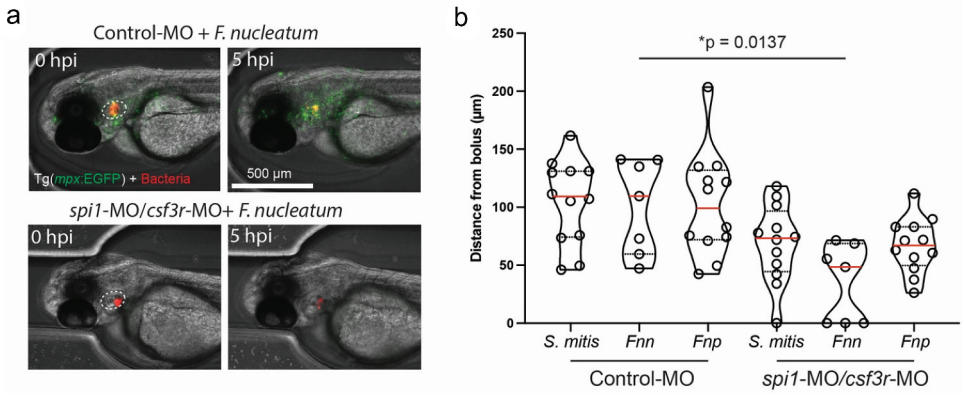

Figure 4.Leukocytes are required for disseminating *Fnn in vivo*. a) Representative images of Control-MO and spi1-MO/csf3r-MO treated larvae 5 hours post-infection (hpi) with *F. nucleatum*. the Control-MO animal has GFP-expressing neutrophils (green fluorescence), which engulf and distribute AleaFluor-594 labeled bacteria (red fluorescence). Animals injected with a spi1-MO /csf3r-MO cocktail have suppressed leukocyte development. Injected bacteria remain confined at the infection bolus in the developing ear (otic vesicle). b) Graph shows the average distance of fluorescent bacteria from the infection bolus for different bacterial species in Control-MO and spi1-MO/csf3r-MO-injected larvae at 5 hpi. Dispersion of *Fnn* was significantly reduced at 5 hpi in the absence of leukocytes. Statistics: One-way ANOVA with Tukey's multiple comparisons test. Each point is the average distance of bacteria from the infection bolus within a fish after 5 hours. Red horizontal line represents the median and the dashed lines represent the first and third quartile.

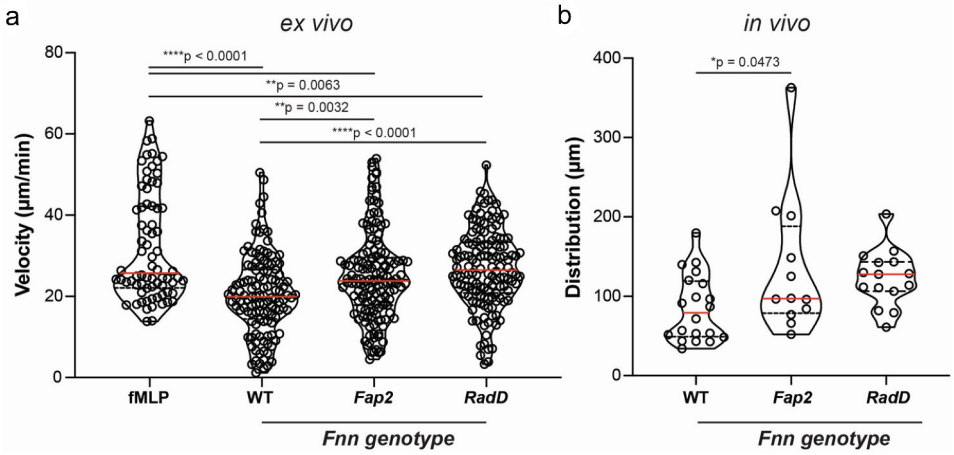

Figure 5. Surface proteins modulate neutrophil dissemination of *Fn nucleatum* ex vivo and in vivo. a) Graph compares the velocity of neutrophils in response to fMLP to cell-laden with wild-type (WT) *Fnn* or *Fnn* with deletion of the Fap2 or RadD genes. Cells laden with mutant bacteria maintained higher velocities than those carrying WT bacteria. Each spot represents the average velocity of a neutrophil track. Data are pooled from $N = 3$ experiments. Red horizontal line represents the median and the dashed lines represent the first and third quartile b) Graph shows that the distribution of zebrafish neutrophils laden with bacteria in vivo at 5 hpi was significantly higher in larvae infected with *Fnn* Fap2 mutant bacteria. Each spot represents the average distribution of laden neutrophils within an infected zebrafish. Data pooled from $N = 3$ experiments. Red horizontal line represents the median and the dashed lines represent the first and third quartile (One-way ANOVA with Tukey's multiple comparisons test.).

microfluidics tools, suggesting that intracellular *Fn* can evade the host's immune response and disseminate away from the oral cavity. The implication of this mechanism of dissemination is that common commensal species of the periodontal microbiome such as *Fn*, can link the oral and systemic pathologies using the host's immune cells. This mechanism is critical for understanding the link between periodontal disease and severe distant pathologies, as it demonstrates that specific bacteria are not directly transported by systemic circulation. We do not know (and did not test) whether the disseminated *Fn* colonizes and forms biofilms in distant organs. However, our observations showed that individual neutrophils could transport *Fn* over distances that could be up to several hundred microns. While this observation may not translate to *in vivo* conditions in humans, in which the distance to various organs is much larger, it supports the neutrophil cell capacity to carry live bacteria. It is possible that neutrophils carry bacteria only to the nearest blood vessels and re-enter the circulation to distribute the bacteria body-wide. Neutrophils have not been implicated in *Fn* dissemination by any mechanism before.

Mutation of bacterial lectin adhesin proteins can dramatically reduce binding, phagocytosis, and killing by neutrophils [58], suggesting that these cell surface proteins can drive antimicrobial activity in host cells. Adhesion to host cells is driven by the binding of the *Fn* Fap2 lectin to host Gal-GalNAc [59]. Previous work on Fap2 and RadD mutants of *Fn* suggested that these outer membrane vesicle proteins induce human lymphocytic cell death [39]. Our findings suggested that these mutants also survive in neutrophils, where Fap2 deletion seems to increase bacterial survival and neutrophil motility substantially. Interestingly, several neutrophil cell-surface (CD43, CD45, C1qRP, and PSGL1) and vesicle-bound (LAMP-1, LAMP-2) receptors are heavily glycosylated, with neutrophil CD43 displaying glycosylation-dependent binding specificity for lectin adhesins expressed by oral species *Streptococcus gordonii* and *Actinomyces naeslundii* [60]. Neutrophil Lamp-1 is enriched in secretory compared to azurophilic granules in neutrophils [61] and has been shown to act as a bacteria-binding receptor [62]. The binding of microbial lectins to proteins such as Lamp-1 may influence intracellular processes following phagocytosis, particularly autophagy, a pathway strongly associated with intracellular survival and Trojan horse dissemination of *S. aureus* by neutrophils [63]. We did not observe any significant reduction in neutrophil viability in these experiments. However, we previously observed and reported that the *Fn* subspecies indeed lead to neutrophil cytotoxicity [38], the highest associated with *Fn polymorphum*. This could be one mechanism that would explain why higher MOIs could be associated with cytotoxicity during longer experiments. In this study, our incubation times were not long. Therefore, RadD-mediated PMN function may be independent of *Fn*-induced PMN viability.

The mechanism described here for neutrophil-mediated bacteria dissemination may apply to other oral bacterial species, such as *P. gingivalis* and *Treponema denticola*, which vary widely in pathogenicity and invasiveness [64,65]. Supporting the

Trojan horse mechanism are studies of pathogens such as *S. aureus* and *Leishmania major*, which were similarly reported to evade intracellular killing in neutrophils, survive, and retain infectivity [66,67]. Taken together, these findings support the microbial dissemination hypothesis in the mechanisms of the link between oral and certain systemic diseases and provide clues for potential therapeutic approaches targeting host-bacteria interactions. The findings from this study will serve as a basis for future studies to probe the mechanisms through which the microbiome of local niches is linked to systemic diseases.

Weaknesses of the zebrafish model include the relatively low-throughput and limited spatial and temporal resolution imposed by the requirement for high-magnification (limited field of view), multichannel, z-stack, and multi-animal time-lapse imaging for these types of experiments. Additionally, the lack of viability readouts for intracellular bacteria for these species, such as GFP expression as used previously [44], limits our interpretation of the data. By combining complementary *in vitro* and *in vivo* models in this study, we have attempted to address the limitations inherent to each.

Additional steps besides the local dissemination would have to be demonstrated. These include the ability of neutrophils carrying microbes to return to blood and later move out of the blood and their chemotaxis towards inflamed distant sites. So far, the ability of neutrophils to reverse their migration and return to blood is supported by evidence in mice, zebrafish, and human neutrophils. In mice, *in vivo* imaging has shown that neutrophils can reverse their migration through the endothelial layer and return from tissues to the blood [68–71]. In zebrafish, neutrophils recruited to the site of wounds have been shown to return to circulation [44,72,73]. Human neutrophils were shown, using microfluidic devices, to migrate out of a blood drop, chemotax towards nano-chambers with chemoattractant, and return to the blood by migrating persistently against gradients [51,73,74]. While several *in vivo* and *in vitro* methods for documenting transmigration and chemotaxis exist, all the steps required for disseminating bacteria by neutrophils toward distant sites could be tested. In addition, how *Fn* escapes the host cells and colonizes at the distant site are critical for the dissemination of the oral bacteria. Our study does not address that issue, but our ongoing studies are designed to elucidate the mechanism through which the bacteria escape the host cells. One critical remaining challenge will be integrating all these steps into a robust model.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Ethics statement

Microinjection of larvae was approved by the Massachusetts General Hospital Subcommittee on Research Animal Care under Protocol 2011N000127. This protocol adheres to the federal Health Research Extension Act and the Public Health Service Policy on the Humane Care and Use of Laboratory Animals, overseen by the National Institutes of Health (NIH) Office of Laboratory Animal Welfare (OLAW).

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