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The emerging oral pathogen, *Filifactor alocis*, extends the functional lifespan of human neutrophils

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Abstract

Periodontitis is a chronic inflammatory infectious disease that affects the integrity of tooth-supporting tissues and has adverse systemic consequences. Advances in sequencing technologies have uncovered organisms that are exclusively found in high numbers in periodontal lesions, such as the gram-positive anaerobic rod, *Filifactor alocis*. *F. alocis* can manipulate neutrophil effector functions, which allows the organism to survive within these granulocytes. Several neutrophil functions have been tested in the context of *F. alocis* challenge, but the effect of the organism on neutrophil apoptosis is still unknown. RNA sequencing of human neutrophils challenged with *F. alocis* showed that apoptosis pathways were differentially regulated. Compared to media-cultured controls, *F. alocis*-challenged neutrophils maintain their nuclear morphology, do not stain for Annexin V or 7-AAD, and have decreased DNA fragmentation. Inhibition of apoptosis by *F. alocis* involved reduced caspase-3, −8, and −9 activation and upregulation of important anti-apoptotic proteins. Prolonged lifespan was dependent on contact through TLR2/6, and *F. alocis*-challenged neutrophils retained their functional capacity to induce inflammation for longer timepoints. This is the first in-depth characterization of neutrophil apoptotic programs in response to an oral pathogen and provides key information on how bacteria manipulate immune cell mechanisms to maintain a dysregulated inflammatory response.

KEYWORDS
apoptosis, *Filifactor alocis*, human neutrophils, periodontitis

1 | INTRODUCTION

Periodontitis is a chronic inflammatory disease where pathogenic microbial communities accumulate in the gingival crevice and cause the loss of alveolar bone and eventually, the tooth (Hajishengallis, 2015). Advances in culture-independent techniques have identified new bacterial species in periodontal lesions, such as the gram-positive anaerobe, *Filifactor alocis* (Deng et al., 2017; Ikeda et al., 2019; Kumar et al., 2003, 2006). Although its full pathogenic potential is not fully characterized, *F. alocis* shares virulence factors with other periodontal pathogens such as the ability to disrupt killing by neutrophils and modulate neutrophil ROS production and NETs generation (Aruni et al., 2011, 2014; Schlafer et al., 2010; Wang et al., 2013, 2014).

Neutrophils are abundant in the bloodstream and the periodontal pocket (Athens et al., 1961; Hsieh et al., 2007). To maintain homeostasis, their large-scale production must be counteracted by efficient removal, since dysregulated neutrophil presence can have devastating consequences (Giammarco et al., 2017; Nauseef, 2007; Summers et al., 2010). Thus, neutrophils undergo apoptosis after ~12–24 h. In tissues, neutrophil lifespan is prolonged by cytokines, microbial components, and the local environment (Kennedy & DeLeo, 2009). Generally, neutrophil viability is promoted by intracellular pathogens as a way to protect their replicative niche, whereas extracellular microbes accelerate neutrophil apoptosis, trigger lysis, or redirect cell death toward necrosis to evade intracellular killing (Allen & Criss, 2019). Little is known about how periodontal pathogens affect neutrophil lifespan during periodontitis. Moreover, defects in neutrophil apoptosis could contribute to the dysregulated inflammation in periodontitis, a possibility that has not been explored in depth.

F. alocis affects neutrophil functional responses (Armstrong et al., 2016; Edmisson et al., 2018; Miralda et al., 2020; Vashishta et al., 2019), and our RNAseq screen of human neutrophils challenged with *F. alocis* showed that apoptosis pathways were a top hit (Miralda et al., 2020). Thus, we conducted a mechanistic study to characterize the effect of *F. alocis* on neutrophil survival. Our results are the first to demonstrate that contact of neutrophils through TLR2/6 with *F. alocis* results in an upregulation of anti-apoptotic proteins, dampening of caspase activity, and decrease in neutrophil apoptosis. Furthermore, *F. alocis*-challenged neutrophils retain their pro-inflammatory effector functions at later timepoints, implicating *F. alocis*-driven neutrophil survival in the sustained dysregulated inflammation of periodontitis.

2 | MATERIALS & METHODS

2.1 | Neutrophil isolation

Human neutrophils were isolated from the venous blood of healthy donors as described previously (Uriarte et al., 2011). In some experiments, further purification by negative magnetic selection was performed using the Easy Eights EasySep Magnet and human neutrophil enrichment kit (Stemcell Technologies, Vancouver, BC, Canada) (Miralda et al., 2020; Vashishta et al., 2019).

2.2 | Bacterial strains and growth conditions

F. alocis ATCC 38596 was cultured, opsonized, labeled with carboxyfluorescein succinimidyl ester (CFSE), and/or heat-killed as previously described (Armstrong et al., 2016, 2018).

2.3 | Cell culture and infection

Neutrophils were cultured at 37°C, 5%CO_2 in RPMI-1640 with L-glutamine and stimulated with Fas L (500 ng/ml), cycloheximide (1 nM, Sigma), staurosporine (1 μM), opsonized *F. alocis* (multiplicity of infection [MOI] 10, 50, 100), or opsonized heat-killed *F. alocis* (MOI 10). After 24 h of culture, cells were processed as cytospins and stained with a Hema-3 Kit or stained with APC Annexin V Apoptosis Detection Kit and 7-AAD (BioLegend, San Diego, CA, USA). Samples were read on a BD FACSCelesta flow cytometer and data were analyzed using FlowJo software (Ashland, OR, USA). In some experiments, neutrophils were pretreated with TLR6 neutralizing antibody (50 μg/ml, Invivogen) or isotype control (Rat IgG,50 μg/ml, Invivogen) for 60 min and/or TLR2 neutralizing antibody (50 μg/ml; clone TL2.1; BioLegend) or isotype control IgG2a kappa (50 μg /ml; clone MOPC-173; BioLegend) for 30 min. All experiments were done in media with no serum except for assays involving conditioned media or transwells where media was supplemented with 5% heat-inactivated fetal bovine serum.

2.4 | RNAseq, data processing, and analysis

Sequencing and analyses were performed as described in (Miralda et al., 2020). Briefly, highly pure neutrophils (>99%) were unstimulated or challenged with opsonized *F. alocis* at a MOI of 10, for 1, 3, or 6 h. After each time point, the cells were harvested and lysed using Trizol (Life Technologies, Carlsbad, CA, USA) and RNA was extracted using the hybrid method of Trizol and RNeasy minikit (Qiagen, Venlo, Netherlands) followed by further purification on a Qiagen column. Total RNASeq libraries were prepared following Illumina's TruSeq Stranded Total RNA LT with the Ribo-Zero Gold library preparation protocol (Illumina Inc., San Diego, CA, USA, Cat# RS-122-2301). Sequencing was performed on an Illumina NextSeq 500 sequencer targeting 50 M 1x75bp reads per sample. Sequences were directly aligned to the *Homo sapiens* reference genome assembly (hg38.fa). Differential expression analysis between each treatment condition (1 h, 3 h, and 6 h) and the control condition was performed using cufflinks cuffdiff2 (version 2.2.1) (Trapnell et al., 2012, 2013). A q-value cutoff ≤ 0.05 with an absolute |log2FC| ≥ 1 was used to determine differential expression.

2.5 | Reverse transcription and quantitative real-time PCR (RT-qPCR)

Total RNA was extracted from highly purified human neutrophils at 1, 3, and 6 h post-bacterial challenge (Miralda et al., 2020; Vashishta et al., 2019). Sequences of the gene-specific primers (Integrated DNA Technologies, Skokie, Illinois, USA) used in this study are listed in Table S1. Results were calculated and expressed as mean normalized expression (MNE) units after GAPDH normalization as previously described (Arruda-Silva et al., 2017).

2.6 | Neutrophil Western blotting

Neutrophils were lysed for 30 min in ice-cold lysis buffer with 4 mM Diisopropyl fluorophosphate (Armstrong et al., 2016). Unless noted, 10–20 μg/μl total cell lysates were separated by 12% SDS-PAGE

and immunoblotted with antibodies for MCL-1 (1:500, Proteintech, Rosemont, IL, USA), XIAP, caspase 8, caspase 9, caspase 3 and β-actin (1:1000, Cell Signaling, Danvers, MA, USA). Secondary antibodies were used at 1:2000 dilution (Cell Signaling, Danvers, MA, USA). The ECL system (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom) or the SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, Waltham, MA, USA) was used to visualize antigen–antibody reactions. Densitometric values were calculated using Image Lab software (BioRad, Hercules, CA, USA).

2.7 | Luminescent caspase activity assays

The activity of caspase 3/7, 8, and 9 was assayed using the Caspase-Glo assay kits from Promega (Madison, WI, USA).

2.8 | Fluorescence caspase 3/7 activity assay

Adherent neutrophils (1×10^6 cells in 500 μl) were untreated or stimulated with opsonized *F. alocis* (MOI 10). The infection was synchronized by a centrifugation step (4 min, 600 g, 14°C) and the cells were cultured for the indicated times. At each timepoint, 1 drop of CellEvent™ Caspase-3/7 Green ReadyProbes™ Reagent (Invitrogen) was added per well for 30 min. The wells were washed with sterile PBS and the cells were fixed with 4% paraformaldehyde. Coverslips were mounted onto glass slides and imaged with a confocal microscope. One hundred cells from throughout the coverslip were counted and evaluated for expression of GFP.

2.9 | Assessment of DNA fragmentation

DNA fragmentation was determined using the ApoBRDU apoptosis detection kit (BD Biosciences, San Jose, CA, USA). After labeling, neutrophils were fixed with 4% paraformaldehyde for 60 min at 4°C, washed, and stained for 90 min.

2.10 | Transwell experiments

Neutrophils were added to the bottom chamber of a 24 well plate with transwells containing a 0.4 μm filter. In top chamber, 100 μl of either media or opsonized live or heat-killed *F. alocis* was added. A separate well had both *F. alocis* and neutrophils in the bottom chamber. After 24 h of culture, duplicates were analyzed for Annexin V/7AAD staining as described above.

2.11 | Generation of conditioned media

Highly pure neutrophils (>99%) were resuspended in colorless RPMI medium supplemented with 5% heat-inactivated human serum (Atlanta Biologicals, Flowery Branch, GA, USA). If needed, neutralizing antibodies were added and after 24 h, supernatants were collected, filter sterilized, aliquoted, and stored at −80°C until use.

2.12 | Imaging flow cytometer assays

Aged neutrophils were challenged with human serum-opsonized, AF488-labeled, *S. aureus* particles (Invitrogen, Carlsbad, CA, USA; MOI 10) in a shaking water bath at 37°C for 30 min. Neutrophils were fixed with 4% paraformaldehyde and their membranes were stained with Alexa Flour 647-conjugated wheat germ agglutinin (2 μg/ml, WGA, Molecular probes, Eugene, OR, USA). For ROS assays, aged neutrophils were incubated with 2′, 7′-dichlorofluorescein (DCF, 5 μM) for 10 min in a shaking water bath at 37°C prior to the *S. aureus* challenge described above. On the IDEAS software, the internalization wizard was used to determine % of *S. aureus* positive cells and the mean fluorescence intensity (MFI) of DCF was quantified for these *S. aureus* positive cells.

2.13 | Statistical analysis

Statistical differences were analyzed using GraphPad Prism Software (Graphpad San Diego, CA, USA). Differences were considered significant when $p<.05$. Specific statistical tests for each experiment are listed in their respective figure legend. If results represent data from multiple experiments, mean values ± standard error of mean (*SEM*) are shown.

3 | RESULTS

3.1 | *F. alocis* extends neutrophil lifespan

Cytospins of freshly isolated cells showed the distinctive multilobular nuclear structure of neutrophils, and culture of neutrophils in media with no serum or culture with pro-apoptotic cycloheximide (CHX) for 24 h resulted in the condensation of nuclei (Figure 1a). Neutrophils co-cultured with *F. alocis* retained their nuclear shape after 24 h. Viability was confirmed by flow cytometry (Figure 1b,c), where *F. alocis*-challenged neutrophils had a greater percentage of viable cells (Q4; $p<.01$ compared to media condition), and smaller percentages of early and late apoptotic cells compared to media (Q3; $p<.05$ and Q2; no significance compared to media condition). Furthermore, cytospin (Figure 1d) and flow cytometry (Figure 1e), showed that the prolonged viability of neutrophils is dose dependent, since using larger multiplicities of infection increased the percentage of cells that retained their nuclear morphology and stained negative for Annexin V and 7-AAD.

To define if *F. alocis* was actively extending neutrophil lifespan, the viability of neutrophils was examined after challenge

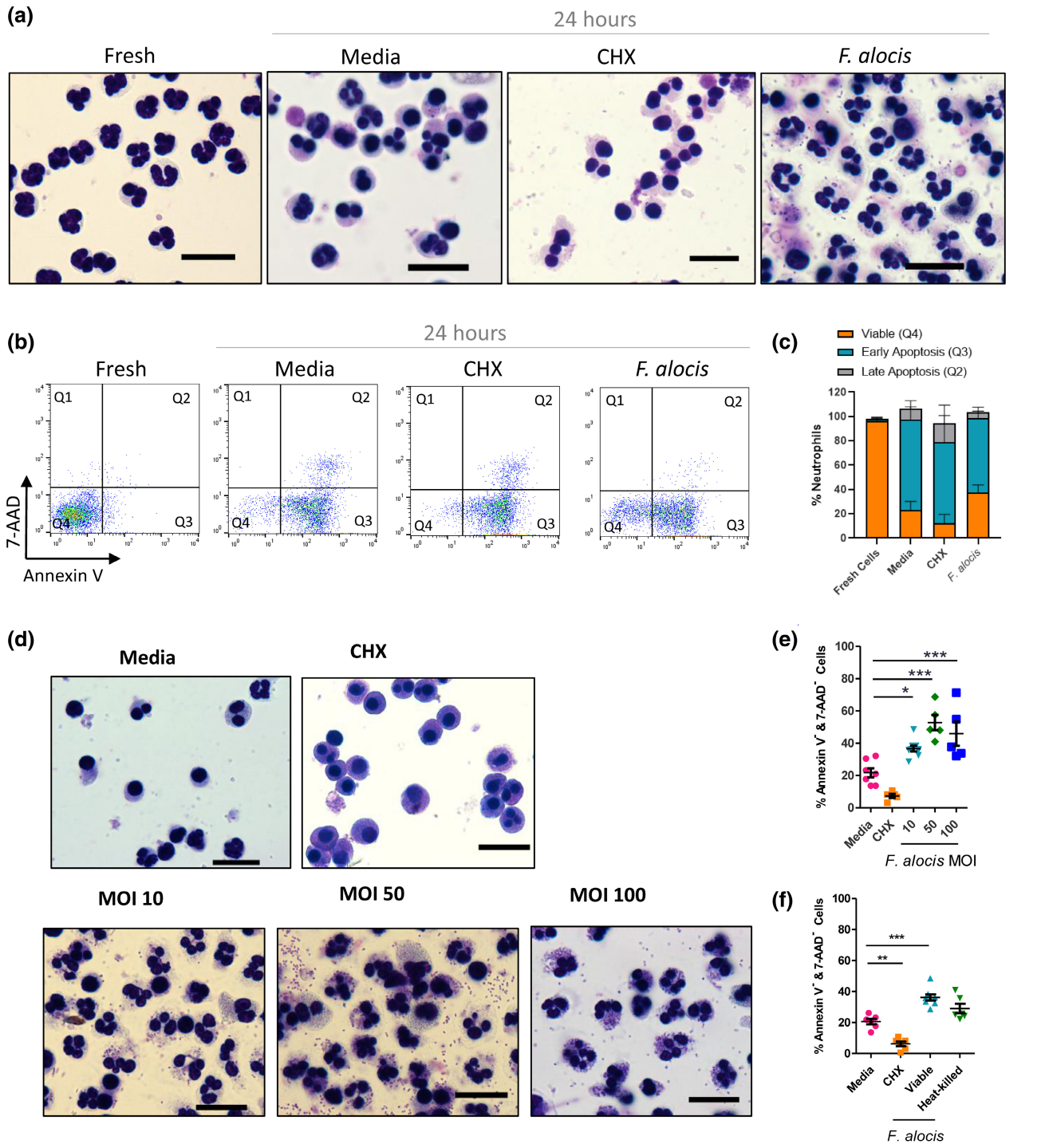

FIGURE 1 *Filifactor alocis* delays neutrophil apoptosis. (a, d) Nuclear morphology changes were assessed by light microscopy using images of Hema-3 stained cytospins from freshly isolated neutrophils, neutrophils cultured in media, or neutrophils stimulated with cycloheximide (CHX), or *F. alocis* (MOI 10, 50, or 100) for 24 h. Scale bar: 20 μm, 100x objective. (b) Flow cytometry dot plot and gating strategy of AnnexinV/7AAD stained neutrophils treated with the same conditions as (a). (c) Shows the % viable (Q4: Annexin V-, 7AAD-), % early apoptotic (Q3: Annexin V+, 7AAD-), and late apoptotic (Q2: Annexin V+, 7AAD+) neutrophils ± *SEM* from two to seven independent experiments. (e, f) Plots show the % viable (Q4: Annexin V-, 7AAD-) neutrophils ± *SEM* from two to seven independent experiments. A one-way ANOVA with Tukey post hoc tests was performed on graphs c, e, and f. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$

with *F. alocis* or heat-killed *F. alocis*. Based on nuclear morphology, both viable and heat-killed *F. alocis* can decrease apoptosis (Data not shown). By flow cytometry, culture of neutrophils with heat-killed *F. alocis* elevated the percentage of cells that were viable, albeit still lower than viable *F. alocis* (Figure 1f). These data indicate that induction of a dose-dependent decrease in the constitutive apoptosis of neutrophils does not depend on the viability of *F. alocis*.

3.2 | *F. alocis* induces changes in the transcriptome to extend neutrophil lifespan

RNAseq data of human neutrophils challenged with *F. alocis* show that 11% of differentially expressed genes (DEG) are functionally categorized under the cellular process of apoptosis (Miralda et al., 2020). After *F. alocis* challenge of human neutrophils, several members of the inhibitor of apoptosis (IAP) family like BIRC8, XIAP, cIAP1 and 2, Livin, NAIP were differentially expressed; and from the extrinsic and intrinsic pathway of apoptosis, caspase 8, FADD, and caspase 9 were downregulated (Table S2, Figure S1).

Based on the transcriptome data, six well-known genes with significant changes in expression from basal were selected (Figure S2) and validated by qPCR (Figure S3). The expression of all genes matched the RNAseq dataset. From the anti-apoptotic proteins: XIAP was significantly upregulated as the time course progressed; cIAP2 was significantly upregulated at 1 h, but decreased by 3 and 6 h; and myeloid cell leukemia 1 (MCL1), had similar expression levels to basal conditions but was downregulated by 3 and 6 h (Figures S2 and S3). As for pro-apoptotic proteins, Bak1 showed a downregulation trend and Bax was significantly upregulated by 6 h. At all timepoints tested, *F. alocis* challenge resulted in a significant downregulation of XK Related protein 8 (XKR8), a caspase-activated scramblase that exposes phosphatidylserine during apoptosis (Suzuki et al., 2016).

3.3 | *F. alocis* induces changes in the expression of pro-survival proteins

Neutrophil survival relies heavily on the expression of MCL-1, an anti-apoptotic member of the Bcl-2 family (Murphy & Caraher, 2015). MCL-1 has a short half-life and requires continuous synthesis to carry out its pro-survival effects; however, the gene expression of MCL1 decreased in *F. alocis*-stimulated cells at later timepoints (Figures S2e and S3e). At the protein level, media-cultured cells had a steady, time-dependent decrease in MCL-1 protein levels, while treating the cells with FasL accelerated MCL-1 degradation (Figure 2a,b). Neutrophils treated with *F. alocis* showed a trend of slower degradation of MCL-1 than media-cultured cells.

XIAP binds to caspase 3 to inhibit its activation and is considered the most potent caspase inhibitor in vitro (Eckelman & Salvesen, 2006). *F. alocis* challenge upregulates XIAP gene expression in human neutrophils (Figures S2a and S3a), which was matched at the protein level (Figure 2c,d). In *F. alocis*-treated conditions, increased XIAP protein expression was detectable at 3 h and remained significantly elevated until 12 h.

3.4 | *F. alocis* dampens caspase 3 activation and decreases DNA fragmentation

Due to the augmented expression of XIAP, we hypothesized that the activation of caspase 3 may be impaired in *F. alocis*-treated

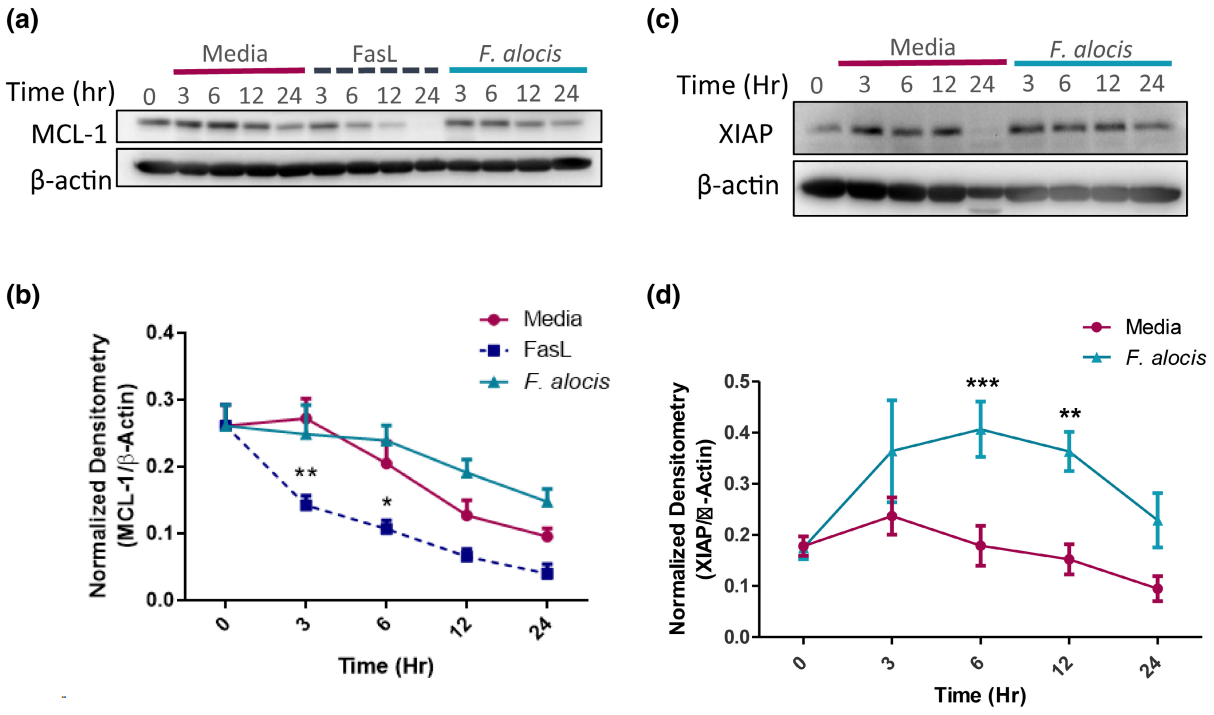

FIGURE 2 *Filifactor alocis* delays the degradation of MCL-1 and upregulates expression of XIAP. Representative images show the immunoblots of whole-cell lysates from media-cultured cells or cells stimulated with FasL or *F. alocis* that were probed for MCL-1 (a) or XIAP (c). Blots were stripped and re-probed with β-actin as a loading control. Normalized immunoblots densitometric values were summarized from seven independent experiments, respectively, and plotted ± *SEM* (b, d). Two-way ANOVA with Bonferroni post hoc testing was performed on all graphs. In (b) one asterisk denotes statistical significance in comparison to media. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$

cells. Western blotting showed that media-treated cells showed a time-dependent decrease in pro-caspase 3 and an increase in the expression of the cleaved caspase 3 fragment, while FasL treatment accelerated the accumulation of cleaved caspase 3 (Figure 3a,b). In contrast, *F. alocis*-challenged neutrophils showed cleaved caspase 3 only after 12 h of bacterial challenge, suggesting that *F. alocis* decreases caspase 3 processing. The activity of caspase 3 was tested in the same experimental conditions by a chemiluminescent assay (Figure 3c) and a fluorescence-based assay (Figure S4a,b). The kinetics of caspase 3 cleavage correlated closely with enzymatic activity and *F. alocis* challenge significantly diminished protease activity as compared to media or FasL stimulated cells.

The final outcome of caspase 3 activation is the destruction of cellular structures like DNA (McIlwain et al., 2013). FasL-stimulated cells had DNA fragmentation starting at 3 h and media-cultured neutrophils became TUNEL positive at 12 h (Figure S5a,b). However, fewer *F. alocis*-stimulated cells were TUNEL positive at both 12 and 24 h. Relative to untreated cells, *F. alocis* markedly decreased DNA fragmentation to retain neutrophil viability.

3.5 | *F. alocis* weakly activates initiator caspases

Since caspase 3 activation is decreased in neutrophils stimulated with *F. alocis*, we determined which pathway is triggered upstream of its activation. Caspase 8 is the initiator caspase of the extrinsic apoptosis pathway (McCracken & Allen, 2014), but western blotting demonstrated that *F. alocis*-challenged neutrophils had no detectable cleaved caspase 8 at any timepoint tested (Figure 4a,b). Using an activity assay, *F. alocis*-challenged cells also did not show any caspase 8 activity at any point in the time course (Figure 4c).

In neutrophils, the extrinsic pathway is not sufficient to induce cell death, and requires the signal to be amplified by the intrinsic pathway (Scaffidi et al., 2000). Thus, we tested the processing and activation of caspase 9, which drives the intrinsic pathway (McCracken & Allen, 2014). The processing of procaspase 9 into the mature form could be detected as early as 6 h in media-cultured neutrophils (Figure 4d,e). FasL-stimulation accelerated the processing of caspase 9, but neutrophils treated with *F. alocis* had minimal processing of caspase 9, even after 24 h of challenge. This was mirrored in an activity assay where media-cultured cells and cells stimulated with the caspase 9 activator, staurosporine, had greater caspase 9 activity as compared to *F. alocis*-challenged neutrophils (Figure 4f). Thus, the processing and activity of caspases 8 and 9 was significantly lower in *F. alocis*-treated cells, suggesting that the bacterium may be influencing the apoptotic cascade at multiple points.

3.6 | *F. alocis* pro-survival effect depends on contact with neutrophils through TLR2/6

Neutrophil programmed cell death can be decreased by host-derived factors such as pro-inflammatory cytokines or bacteria-derived components. Thus, using transwells, we tested whether bacterial

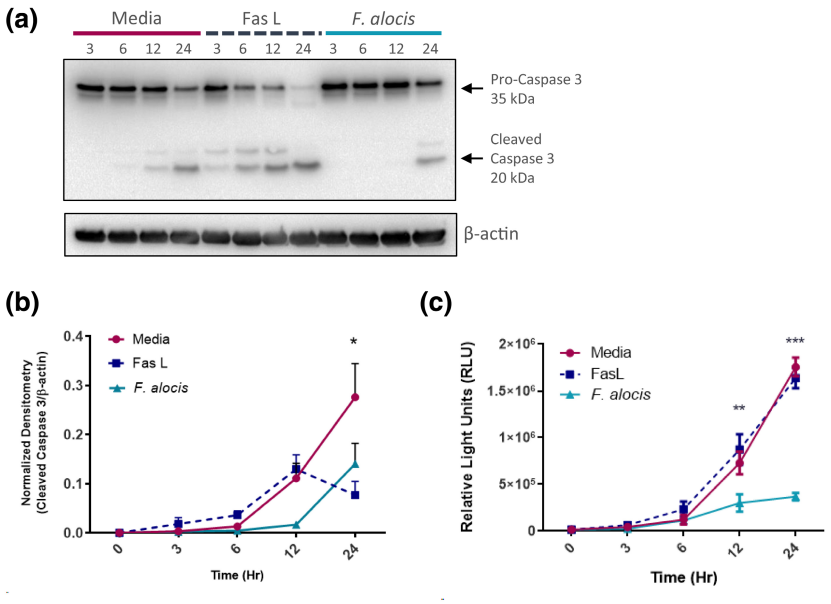

FIGURE 3 *Filifactor alocis* delays caspase 3 processing (a) Representative immunoblot of time-dependent caspase 3 processing from whole-cell lysates of unstimulated (media), FasL or *F. alocis* stimulated neutrophils. Blots were stripped and re-probed with β-actin as a loading control (b) Data are plotted as the mean ± *SEM* of the normalized densitometry of cleaved caspase 3/β-actin from eight independent experiments. Asterisk denotes statistical significance in comparison to both *F. alocis* and FasL. (c) Caspase 3 activity was tested in suspension using a chemiluminescent assay on neutrophils that were unstimulated (Media) or stimulated with FasL or *F. alocis* (MOI 10) for 3, 6, 12, or 24 h. Data are plotted as the mean ± *SEM* of the luminescence produced for each condition from four independent experiments. Asterisk denotes statistical significance between media and FasL compared to *F. alocis* conditions. A two-way ANOVA with Bonferroni post hoc testing was performed on all graphs. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$

contact is required for the prolonged neutrophil lifespan (Figure 5a). When neutrophils and viable *F. alocis* were in the same compartment, viability increased as previously shown (Figure 1b). This effect was reversed when the organism and neutrophils were separated by the transwell filter, confirming that contact is essential to induce the pro-survival phenotype. Neutrophils were also incubated in the same and opposite sides from heat-killed *F. alocis* to assess whether the bacteria were actively releasing pro-survival factors. Confirming the findings in Figure 1f, neutrophils co-cultured with heat-killed *F. alocis* showed enhanced survival as compared to unstimulated neutrophils (Figure 5b). Separation of the bacteria and neutrophils abrogated the prolonged survival phenotype, albeit less robustly as the viable bacteria condition.

F. alocis ligation of TLR2/6 initiates numerous neutrophil effector functions (Armstrong et al., 2016; Vashishta et al., 2019); thus, we evaluated whether the apoptosis decrease was mediated through TLR2/6 (Figure 5c). Neutrophils were unstimulated or pretreated with neutralizing antibodies against TLR2 and−6 or corresponding isotype controls prior to stimulation with *F. alocis* for 24 h. Blocking access to TLR2/6 by antibodies decreased the viability of *F. alocis*-challenged neutrophils comparable to basal levels, while the isotype control did not have an effect. Combined, these data demonstrate that *F. alocis* contact with neutrophils through TLR2/6 ligation is responsible for the prolonged neutrophil lifespan.

3.7 | Factors released during *F. alocis* & neutrophil interaction are pro-survival

The pro-survival response we observed could be due to a paracrine effect. Therefore, supernatants of neutrophils cultured in media or stimulated with *F. alocis* were collected and sterile-filtered to generate conditioned media (Figure 6a). Naïve neutrophils were cultured in fresh or conditioned media for 24 h and their apoptosis status was assessed (Figure 6b,c). Neutrophils cultured in conditioned media from untreated cells developed nuclear condensation, whereas the multi-lobulated nuclear morphology was retained when neutrophils were cultured in the conditioned media from *F. alocis*-stimulated

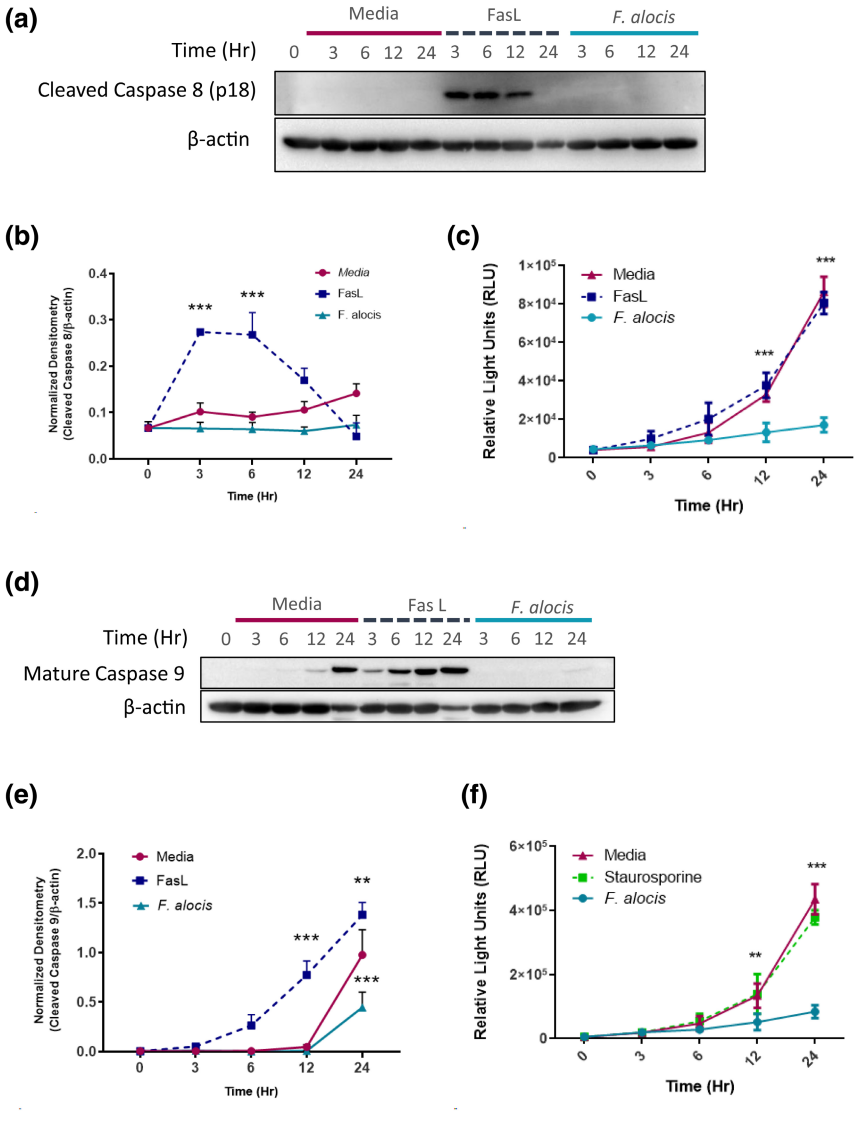

FIGURE 4 *Filifactor alocis* minimally activates the initiator caspases, 8 and 9. Caspase 8 (a) and caspase 9 (d) processing was assessed by quantifying immunoblots for the active, cleaved portion of the proteases in the whole-cell lysates of cells cultures in media, with FasL, or opsonized *F. alocis*. Blots were stripped and re-probed with β-actin as loading control. Data are plotted as the average normalized densitometry ± *SEM* from three to five independent experiments for caspase 8 (b) and caspase 9 (e), respectively. Next, activity of caspase 8 (c) and caspase 9 (f) was tested using a chemiluminescent activity assay on neutrophils that were unstimulated (Media), stimulated with positive controls FasL or staurosporine, or *F. alocis* (MOI 10) for 3, 6, 12, or 24 h. Data are plotted as the mean ± *SEM* of the luminescence produced for each condition from four independent experiments. Two-way ANOVA with Bonferroni post hoc testing was performed on b, c, e, and f where $^{**}p < .01$, $^{***}p < .001$

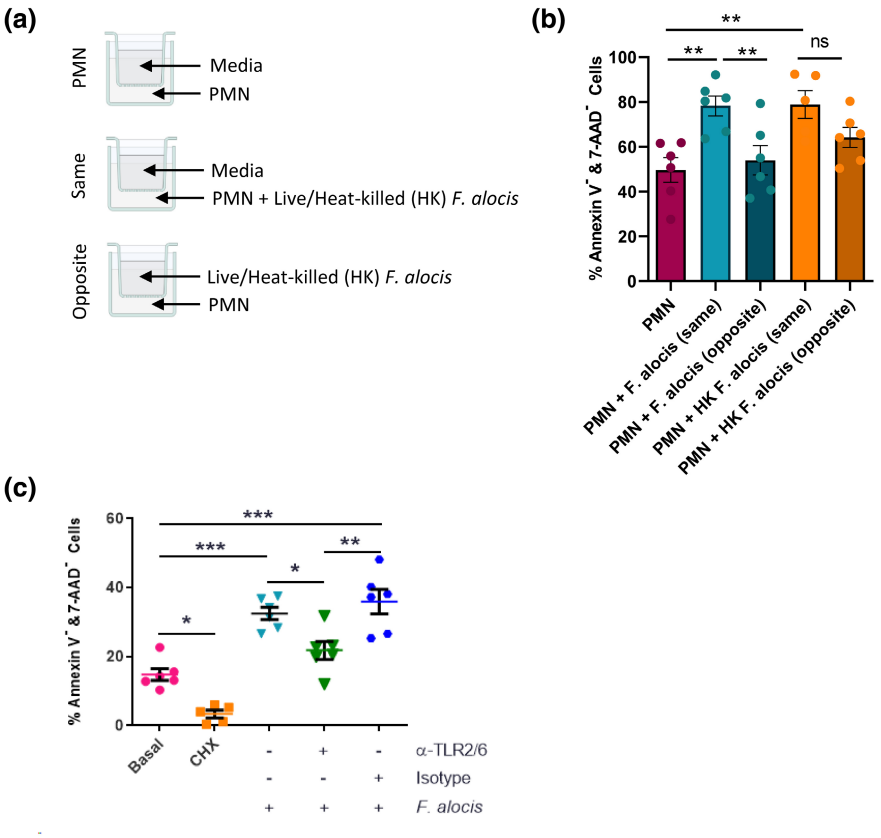

FIGURE 5 Contact with *Filifactor alocis* through TLR2/6 is necessary for prolonged neutrophil lifespan. A diagram shows the experimental layout used with the transwells system. Neutrophils (PMN) were always placed in the bottom chamber of the transwells alone (PMN), in contact with live or heat-killed *F. alocis* (Same), or alone but the bacterium was placed on the upper chamber (Opposite) for 24 h followed by Annexin V/7AAD staining analyzed by flow cytometry (a). Data are plotted as the mean percent viable (Q4: Annexin V-, 7AAD-) neutrophils ± *SEM* from six independent experiments (b). Neutrophils were unstimulated (Basal), stimulated with cyclohexamide (CHX), stimulated with *F. alocis* in the presence or absence of neutralizing TLR2/6 antibodies or isotype controls for 24 h. Data are plotted as the mean percent viable (Q4: Annexin V-, 7AAD-) neutrophils ± *SEM* from five to six independent experiments (c). A one-way ANOVA with Tukey post hoc testing was performed on (b) and (c) to determine statistical significance. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$

cells. Neutrophils in the *F. alocis*-conditioned media had increased viability when compared to untreated cells, indicating that the interaction between *F. alocis* and neutrophils generates pro-survival factors (Figure 6c). However, the percentage of viable cells was not as robust as when neutrophils are stimulated with *F. alocis* in fresh media, suggesting that both the bacteria-neutrophil interaction and the secreted pro-survival factors are necessary to induce maximal neutrophil survival.

To determine the contribution of TLR2/6 signaling to pro-survival factor generation, a new set of conditioned media was collected where neutrophils were pretreated with neutralizing antibodies against TLR2/6 or isotype controls prior to *F. alocis* stimulation. The supernatants were used to culture naïve neutrophils for 24 h (Figure 6d). While stimulating neutrophils with *F. alocis* yields the highest percentage of viable neutrophils, culturing cells with *F. alocis*-conditioned media still maintain higher viability than untreated cells. When neutrophils were cultured with the conditioned media from neutrophils pretreated with inhibitors for TLR2/6, the pro-survival effect is abrogated, suggesting the ligation of TLR2/6 initiates a signaling cascade that produces pro-survival factors with paracrine effects on noninfected cells. Notably, this pro-survival milieu is not sufficient to elicit the maximal neutrophil survival possible in this context and requires the presence of the bacterium.

3.8 | Neutrophils with *F. alocis*-mediated survival retain their functional capacity

Neutrophils undergoing apoptosis have restricted proinflammatory capacities in regards to chemotaxis, phagocytosis, and degranulation (McCracken & Allen, 2014). *F. alocis* challenge extends neutrophil viability; thus, we hypothesized that the functional capacity *F. alocis*-cultured neutrophils would also be retained. Neutrophils were aged in media or media with opsonized *F. alocis* for 1, 18, or 24 h, followed by stimulation with nonviable *S. aureus* particles because they are known to be ingested easily and induce robust ROS generation. Figure 7a shows representative images of neutrophils that were classified as *S. aureus* negative or *S. aureus* positive after imagestream analysis. After 1 h of culture, both media and *F. alocis* cultured neutrophils were equally efficient at engulfing the *S. aureus* (Figure 7b).

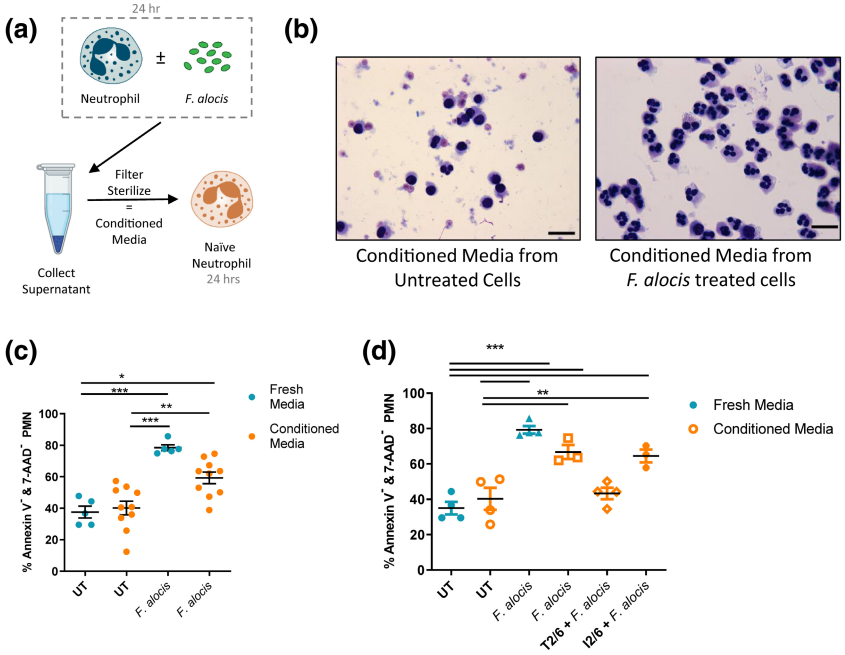

FIGURE 6 Pro-survival factors are released during the *Filifactor alocis* & neutrophil interaction. (a) Graphic depicting how conditioned media were generated. (b) Hema-3 stained cytospins of naïve neutrophils cultured for 24 h in the conditioned media collected from untreated cells or *F. alocis*-treated cells. Scale bar: 20 μm, 100 x objective. (c) Neutrophils were cultured in fresh media and left untreated (UT) or stimulated with *F. alocis* (blue dots), or cultured in the conditioned media collected of untreated (UT) of *F. alocis*-stimulated cells for 24 h (orange dots); followed by staining for Annexin V and 7AAD and samples analyzed by flow cytometry. Data are plotted as the mean percent viable (Q4: Annexin V-, 7AAD-) neutrophils ± *SEM* from 5 to 10 independent experiments. (d) Naïve neutrophils were cultured in fresh media and left untreated (UT) or stimulated with *F. alocis* (depicted in blue), or cells were cultured in the conditioned media from untreated cells or cells stimulated with *F. alocis* in the presence or absence of neutralizing TLR2/6 antibodies or isotype controls (depicted in orange). After culture for 24 h, the cells were stained for Annexin V and 7AAD and analyzed by flow cytometry. Data are plotted as the mean percent viable (Q4: Annexin V-, 7AAD-) neutrophils ± *SEM* from five independent experiments. One-way ANOVA with Tukey post hoc testing was performed on (c) and (d) to determine statistical significance. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$

After aging for 18 h, *F. alocis*-challenged neutrophils retain the ability to phagocytize *S. aureus* compared to noninfected cells. Neutrophils from the same donors were also tested after 24 h, and while the trend was the same, there was no statistical significance between media-aged neutrophils and their *F. alocis*-challenged counterparts.

The ability of aged neutrophils to produce ROS was also assessed by imaging flow cytometry (Figure 7c). At 1 h, there is no difference in the basal ROS generation between media or *F. alocis*-cultured neutrophils (Figure 7d). When *S. aureus* is added to the neutrophils, the ROS generation in both conditions becomes significantly higher compared to baseline, although *F. alocis*-treated neutrophils produce more ROS than the media cultured controls (Figure 7d). This difference is likely due to neutrophil priming by *F. alocis*, which has been previously described (Edmisson et al., 2018). After 18 h, untreated cells lose some of their capacity to produce ROS against *S. aureus*, whereas *F. alocis* stimulated cells produced significantly higher ROS (Figure 7c,d). This difference was lost at 24 h, where *F. alocis*-cultured cells also begin to lose their functional capacity. Thus, *F. alocis*-challenged neutrophils also retain their ability to produce ROS upon subsequent challenge longer than unstimulated cells. Ultimately, the prolonged lifespan and functional capacity of neutrophils induced by *F. alocis* could result in increased neutrophil-driven inflammation, which is a hallmark of periodontal disease.

4 | DISCUSSION

Neutrophils undergo apoptosis to diminish their pro-inflammatory potential and become targets for efferocytic clearance. This initiates inflammation resolution protocols and tissue repair that are essential for effective immune responses (Hochreiter-Hufford & Ravichandran, 2013). Neutrophils accumulate extensively in periodontitis, where they are found in different stages of cell death (Crawford et al., 2000; Gamonal et al., 2003; Nicu et al., 2018). This suggests that there is modulation of neutrophil lifespan and/or a defect in their efficient clearing. The few studies addressing neutrophil viability after encounters with oral bacteria focused on short timepoints (3 h or less) where the extent of their effect on neutrophil viability cannot be fully appreciated (Jewett et al., 2000; Kurgan et al., 2017; Permpanich et al., 2006; Shin et al., 2008). *F. alocis* challenge induces apoptosis in normal oral keratinocytes and epithelial cells (Chioma et al., 2017; Wang et al., 2014); contrastingly, *F. alocis*

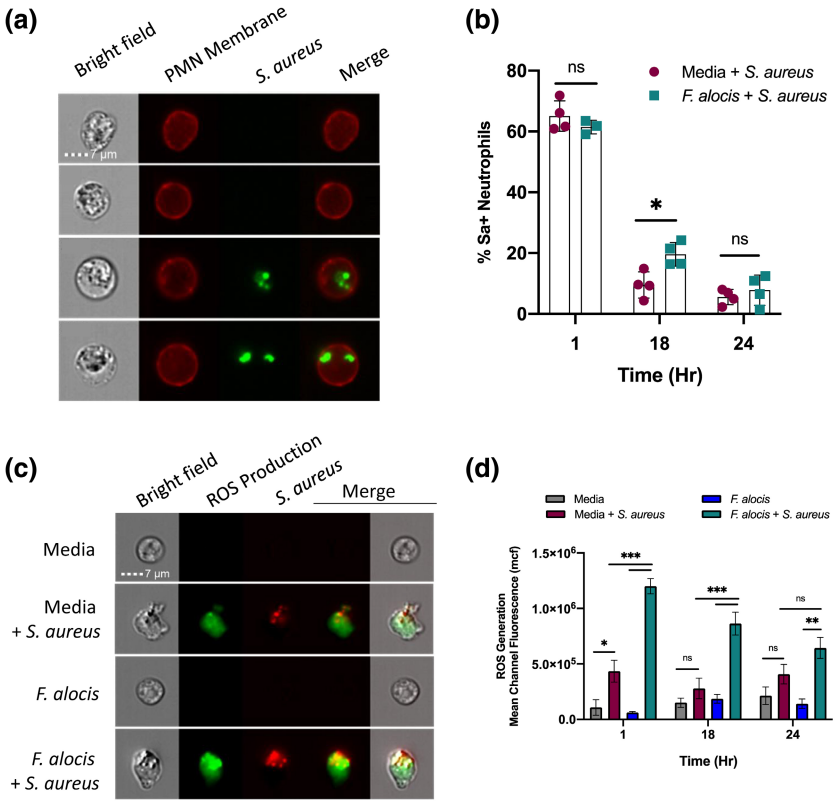

FIGURE 7 *Filifactor alocis*-challenged neutrophils retain effector functions for longer timepoints. Neutrophils were aged for 1, 18, or 24 h in media or with *F. alocis* followed by a 30-min challenge with AlexaFluor 488 labeled—*S. aureus*. First, the phagocytic capacity of these cells was tested via imaging flow cytometry. (a) Representative images from the imaging flow cytometer depict neutrophils (neutrophil membrane was labeled with WGA depicted in red) that did or did not internalize *S. aureus* (green). (b) Summary from four independent experiments shows the mean percentage of neutrophils that phagocytosed *S. aureus*. (c) Representative images from the imaging flow cytometer show the ROS production in response to *S. aureus* challenge by cells aged for 18 h with media or *F. alocis*. (d) Average MFI of DCF positive ± *SEM* from media-aged cells or *F. alocis*-aged cells that internalized *S. aureus* from four independent experiments. Two-way ANOVA with Bonferroni post hoc testing was performed on (b) and (d) to determine statistical significance. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$

extends neutrophils' lifespan, which depends on bacterial contact and TLR2/6 signaling.

We used serum-free media in our experimental design to eliminate the confounding effects of growth factors and serum components (Gardai et al., 2004; Schwartz et al., 2012). Even in these limiting conditions, *F. alocis* decreased neutrophil constitutive apoptosis pathways. Relative to control cells, neutrophils challenged with *F. alocis* retained their nuclear morphology, showed decreased phosphatidylserine externalization, and had diminished DNA fragmentation. This phenotype could be explained by a marked decrease in the processing and subsequent activity of the executioner caspase 3; an effect that has been previously observed for other intracellular pathogenic bacteria such as *Francisella tularensis* (Schwartz et al., 2012). It is likely that in the case of *F. alocis*-challenged neutrophils, the decreased processing of caspase 3 is partially linked to increased expression of XIAP, a protein that physically binds to caspase 3 and prevents its activation. This is similar to the mechanism deployed by *Neisseria gonorrhoeae*, which transiently decreases the onset of apoptosis by upregulating cIAP2 and XIAP (Simons et al., 2006). The relationship between the relative abundance of pro-survival versus proapoptotic factors is a key determinant that regulates neutrophils' fate. In our study, *F. alocis* induced a significant time-dependent increase in mRNA levels of the pro-survival factor XIAP. Up until 12 h *F. alocis* prevented XIAP degradation, with levels decreasing by 24 h but remaining higher compared to unstimulated cells. We can speculate that by preventing XIAP degradation, more than MCL-1, *F. alocis* impairs activation of caspase 9 and 3 to extend neutrophil lifespan.

At 6 h, XIAP levels decrease, procaspase 3 is cleaved, and DNA fragmentation becomes detectable. While this suggests that viable *F. alocis* may be actively inducing XIAP expression, heat-killed *F. alocis* also had a pro-survival phenotype. This was surprising since heat-killed *F. alocis* results in efficient phagosome maturation and induces robust ROS production (Edmisson et al., 2018) that initiates the phagocytosis-induced cell death (PICD) program. Our results also demonstrated that the pro-survival effect requires contact with *F. alocis*. Both viable and heat-killed *F. alocis* activate TLR2/6, which was necessary to induce neutrophil survival. Conditioned media from neutrophils stimulated with *F. alocis* had pro-survival effects on naïve neutrophils, likely through pro-inflammatory cytokines that extend paracrine neutrophil survival effects.

Neutrophil cell death benefits bacteria by preventing phagocytosis and microbicidal mechanisms involved in their elimination. When neutrophil apoptosis is decreased in periodontitis, inflammophilic bacteria profit as inflammation worsens by two mechanisms: (1) collateral tissue damage increases when neutrophils accumulate and continue releasing degradative enzymes and ROS, and (2) resolution of inflammation is delayed due to the lack of efferocytotic clearance of apoptotic cells, which is essential for the initiation of tissue restoration mechanisms. In conclusion, this is the first in-depth characterization of the effect the emerging oral pathogen, *F. alocis*, has on human neutrophils' apoptosis. Although more work will be needed to establish the in vivo relevance of this data, we speculate that the extended neutrophil lifespan will have serious implications in periodontitis progression by extending the window of time where neutrophils can promote dysbiotic inflammation in the oral cavity.

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CONFLICT OF INTEREST

The authors declare no competing financial interest.

AUTHORS' CONTRIBUTION

IM performed the majority of the experiments and wrote the manuscript draft. AV performed the qPCR experiments. MR performed the imaging flow cytometry experiments. RJL provided valuable scientific insights and helped in the writing and edits of the manuscript. SMU provided the financial support, study design and was involved in the writing and edits of the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Human donor recruitment, blood draws, and materials were in accordance with the guidelines approved by the Institutional Review Board of the University of Louisville. Informed consent was obtained from all participating subjects from approved IRB#96.0191.

DATA AVAILABILITY STATEMENT

RNAseq dataset can be found in the GEO at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi under accession number GSE137351. The data that support the findings of this study are available from the corresponding author upon reasonable request.

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REFERENCES

Allen, L.H. & Criss, A.K. (2019) Cell intrinsic functions of neutrophils and their manipulation by pathogens. *Current Opinion in Immunology*, *60*, 124–129.

Armstrong, C.L., Klaes, C.K., Vashishta, A., Lamont, R.J. & Uriarte, S.M. (2018) *Filifactor alocis* manipulates human neutrophils affecting their ability to release neutrophil extracellular traps induced by PMA. *Innate Immunity*, *24*(4), 210–220.

Armstrong, C.L., Miralda, I., Neff, A.C., Tian, S., Vashishta, A., Perez, L. et al. (2016) *Filifactor alocis* promotes neutrophil degranulation and chemotactic activity. *Infection and Immunity*, *84*(12), 3423–3433.

Arruda-Silva, F., Bianchetto-Aguilera, F., Gasperini, S., Polletti, S., Cosentino, E., Tamassia, N. et al. (2017) Human neutrophils produce CCL23 in response to various TLR-agonists and TNFalpha. *Frontiers in Cellular and Infection Microbiology*, *7*, 176.

Aruni, A.W., Roy, F. & Fletcher, H.M. (2011) *Filifactor alocis* has virulence attributes that can enhance its persistence under oxidative stress conditions and mediate invasion of epithelial cells by *Porphyromonas gingivalis*. *Infection and Immunity*, *79*(10), 3872–3886.

Aruni, A.W., Zhang, K., Dou, Y. & Fletcher, H. (2014) Proteome analysis of coinfection of epithelial cells with *Filifactor alocis* and *Porphyromonas gingivalis* shows modulation of pathogen and host regulatory pathways. *Infection and Immunity*, *82*(8), 3261–3274.

Athens, J.W., Haab, O.P., Raab, S.O., Mauer, A.M., Ashenbrucker, H., Cartwright, G.E. et al. (1961) Leukokinetic studies. IV. The total blood, circulating and marginal granulocyte pools and the granulocyte turnover rate in normal subjects. *Journal of Clinical Investigation*, *40*, 989–995.

Chioma, O., Aruni, A.W., Milford, T.A. & Fletcher, H.M. (2017) *Filifactor alocis* collagenase can modulate apoptosis of normal oral keratinocytes. *Molecular Oral Microbiology*, *32*(2), 166–177.

Crawford, J.M., Wilton, J.M. & Richardson, P. (2000) Neutrophils die in the gingival crevice, periodontal pocket, and oral cavity by necrosis and not apoptosis. *Journal of Periodontology*, *71*(7), 1121–1129.

Deng, Z.L., Szafranski, S.P., Jarek, M., Bhuju, S. & Wagner-Dobler, I. (2017) Dysbiosis in chronic periodontitis: key microbial players and interactions with the human host. *Scientific Reports*, *7*(1), 3703.

Eckelman, B.P. & Salvesen, G.S. (2006) The human anti-apoptotic proteins cIAP1 and cIAP2 bind but do not inhibit caspases. *Journal of Biological Chemistry*, *281*(6), 3254–3260.

Edmisson, J.S., Tian, S., Armstrong, C.L., Vashishta, A., Klaes, C.K., Miralda, I. et al. (2018) *Filifactor alocis* modulates human neutrophil antimicrobial functional responses. *Cellular Microbiology*, *20*(6), e12829.

Gamonal, J., Sanz, M., O'Connor, A., Acevedo, A., Suarez, I., Sanz, A. et al. (2003) Delayed neutrophil apoptosis in chronic periodontitis patients. *Journal of Clinical Periodontology*, *30*(7), 616–623.

Gardai, S.J., Whitlock, B.B., Xiao, Y.Q., Bratton, D.B. & Henson, P.M. (2004) Oxidants inhibit ERK/MAPK and prevent its ability to delay neutrophil apoptosis downstream of mitochondrial changes and at the level of XIAP. *Journal of Biological Chemistry*, *279*(43), 44695–44703.

Giammarco, S., Chiusolo, P., Piccirillo, N., Di Giovanni, A., Metafuni, E., Laurenti, L. et al. (2017) Hyperleukocytosis and leukostasis: management of a medical emergency. *Expert Review of Hematology*, *10*(2), 147–154.

Hajishengallis, G. (2015) Periodontitis: from microbial immune subversion to systemic inflammation. *Nature Reviews Immunology*, *15*(1), 30–44.

Hochreiter-Hufford, A. & Ravichandran, K.S. (2013) Clearing the dead: apoptotic cell sensing, recognition, engulfment, and digestion. *Cold Spring Harbor Perspectives in Biology*, *5*(1), a008748.

Hsieh, M.M., Everhart, J.E., Byrd-Holt, D.D., Tisdale, J.F. & Rodgers, G.P. (2007) Prevalence of neutropenia in the U.S. population: age, sex, smoking status, and ethnic differences. *Annals of Internal Medicine*, *146*(7), 486–492.

Ikeda, E., Shiba, T., Ikeda, Y., Suda, W., Nakasato, A., Takeuchi, Y. et al. (2019) Japanese subgingival microbiota in health vs disease and their roles in predicted functions associated with periodontitis. *Odontology*, *108*, 280–291.

Jewett, A., Hume, W.R., Le, H., Huynh, T.N., Han, Y.W., Cheng, G. et al. (2000) Induction of apoptotic cell death in peripheral blood mononuclear and polymorphonuclear cells by an oral

bacterium, *Fusobacterium nucleatum*. *Infection and Immunity*, *68*(4), 1893–1898.
Kennedy, A.D. & DeLeo, F.R. (2009) Neutrophil apoptosis and the resolution of infection. *Immunologic Research*, *43*(1–3), 25–61.
Kumar, P.S., Griffen, A.L., Barton, J.A., Paster, B.J., Moeschberger, M.L. & Leys, E.J. (2003) New bacterial species associated with chronic periodontitis. *Journal of Dental Research*, *82*(5), 338–344.
Kumar, P.S., Leys, E.J., Bryk, J.M., Martinez, F.J., Moeschberger, M.L. & Griffen, A.L. (2006) Changes in periodontal health status are associated with bacterial community shifts as assessed by quantitative 16S cloning and sequencing. *Journal of Clinical Microbiology*, *44*(10), 3665–3673.
Kurgan, S., Kansal, S., Nguyen, D., Stephens, D., Koroneos, Y., Hasturk, H. et al. (2017) Strain-specific impact of *Fusobacterium nucleatum* on neutrophil function. *Journal of Periodontology*, *88*(4), 380–389.
McCracken, J.M. & Allen, L.A. (2014) Regulation of human neutrophil apoptosis and lifespan in health and disease. *Journal of Cell Death*, *7*, 15–23.
McIlwain, D.R., Berger, T. & Mak, T.W. (2013) Caspase functions in cell death and disease. *Cold Spring Harbor Perspectives in Biology*, *5*(4), a008656.
Miralda, I., Vashishta, A., Rogers, M.N., Rouchka, E.C., Li, X., Waigel, S. et al. (2020) Whole transcriptome analysis reveals that *Filifactor alocis* modulates TNFalpha-stimulated MAPK activation in human neutrophils. *Frontiers in Immunology*, *11*, 497.
Murphy, M.P. & Caraher, E. (2015) Mcl-1 is vital for neutrophil survival. *Immunologic Research*, *62*(2), 225–233.
Nauseef, W.M. (2007) How human neutrophils kill and degrade microbes: an integrated view. *Immunological Reviews*, *219*, 88–102.
Nicu, E.A., Rijkschroeff, P., Wartewig, E., Nazmi, K. & Loos, B.G. (2018) Characterization of oral polymorphonuclear neutrophils in periodontitis patients: a case–control study. *BMC Oral Health*, *18*(1), 149.
Permpanich, P., Kowolik, M.J. & Galli, D.M. (2006) Resistance of fluorescent-labelled *Actinobacillus actinomycetemcomitans* strains to phagocytosis and killing by human neutrophils. *Cellular Microbiology*, *8*(1), 72–84.
Scaffidi, C., Kischkel, F.C., Krammer, P.H. & Peter, M.E. (2000) Analysis of the CD95 (APO-1/Fas) death-inducing signaling complex by high-resolution two-dimensional gel electrophoresis. *Methods in Enzymology*, *322*, 363–373.
Schlafer, S., Riep, B., Griffen, A.L., Petrich, A., Hubner, J., Berning, M. et al. (2010) *Filifactor alocis*—involvement in periodontal biofilms. *BMC Microbiology*, *10*, 66.
Schwartz, J.T., Barker, J.H., Kaufman, J., Fayram, D.C., McCracken, J.M. & Allen, L.A. (2012) *Francisella tularensis* inhibits the intrinsic and extrinsic pathways to delay constitutive apoptosis and prolong human neutrophil lifespan. *Journal of Immunology*, *188*(7), 3351–3363.
Shin, J., Ji, S. & Choi, Y. (2008) Ability of oral bacteria to induce tissue-destructive molecules from human neutrophils. *Oral Diseases*, *14*(4), 327–334.
Simons, M.P., Nauseef, W.M., Griffith, T.S. & Apicella, M.A. (2006) *Neisseria gonorrhoeae* delays the onset of apoptosis in polymorphonuclear leukocytes. *Cellular Microbiology*, *8*(11), 1780–1790.
Summers, C., Rankin, S.M., Condliffe, A.M., Singh, N., Peters, A.M. & Chilvers, E.R. (2010) Neutrophil kinetics in health and disease. *Trends in Immunology*, *31*(8), 318–324.
Suzuki, J., Imanishi, E. & Nagata, S. (2016) Xkr8 phospholipid scrambling complex in apoptotic phosphatidylserine exposure. *Proceedings of the National Academy of Sciences of the United States of America*, *113*(34), 9509–9514.
Trapnell, C., Hendrickson, D.G., Sauvageau, M., Goff, L., Rinn, J.L. & Pachter, L. (2013) Differential analysis of gene regulation at transcript resolution with RNA-seq. *Nature Biotechnology*, *31*(1), 46–53.
Trapnell, C., Roberts, A., Goff, L., Pertea, G., Kim, D., Kelley, D.R. et al. (2012) Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nature Protocols*, *7*(3), 562–578.
Uriarte, S.M., Rane, M.J., Luerman, G.C., Barati, M.T., Ward, R.A., Nauseef, W.M. et al. (2011) Granule exocytosis contributes to priming and activation of the human neutrophil respiratory burst. *Journal of Immunology*, *187*(1), 391–400.
Vashishta, A., Jimenez-Flores, E., Klaes, C.K., Tian, S., Miralda, I., Lamont, R.J. et al. (2019) Putative periodontal pathogens, *Filifactor alocis* and *Peptoanaerobacter stomatis*, induce differential cytokine and chemokine production by human neutrophils. *Pathogens*, *8*(2), 59.
Wang, Q., Jotwani, R., Le, J., Krauss, J.L., Potempa, J., Coventry, S.C. et al. (2014) *Filifactor alocis* infection and inflammatory responses in the mouse subcutaneous chamber model. *Infection and Immunity*, *82*(3), 1205–1212.
Wang, Q., Wright, C.J., Dingming, H., Uriarte, S.M. & Lamont, R.J. (2013) Oral community interactions of *Filifactor alocis* in vitro. *PLoS One*, *8*(10), e76271.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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