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Enterococcus faecalis suppresses *Staphylococcus aureus*-induced NETosis and promotes bacterial survival in polymicrobial infections

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Abstract

Enterococcus faecalis is an opportunistic pathogen that is frequently co-isolated with other microbes in wound infections. While *E. faecalis* can subvert the host immune response and promote the survival of other microbes via interbacterial synergy, little is known about the impact of *E. faecalis*-mediated immune suppression on co-infecting microbes. We hypothesized that *E. faecalis* can attenuate neutrophil-mediated responses in mixed-species infection to promote survival of the co-infecting species. We found that neutrophils control *E. faecalis* infection via phagocytosis, ROS production, and degranulation of azurophilic granules, but it does not trigger neutrophil extracellular trap formation (NETosis). However, *E. faecalis* attenuates *Staphylococcus aureus*-induced NETosis in polymicrobial infection by interfering with citrullination of histone, suggesting *E. faecalis* can actively suppress NETosis in neutrophils. Residual *S. aureus*-induced NETs that remain during co-infection do not impact *E. faecalis*, further suggesting that *E. faecalis* possess mechanisms to evade or survive NET-associated killing mechanisms. *E. faecalis*-driven reduction of NETosis corresponds with higher *S. aureus* survival, indicating that this immunomodulating effect could be a risk factor in promoting the virulence polymicrobial infection. These findings highlight the complexity of the immune response to polymicrobial infections and suggest that attenuated pathogen-specific immune responses contribute to pathogenesis in the mammalian host.

Keywords: *Enterococcus faecalis*; *Staphylococcus aureus*; neutrophils; polymicrobial infection; neutrophil extracellular traps

Introduction

Enterococci are one of the leading pathogens associated with skin and soft tissue infections (SSTIs), particularly surgical site infections and diabetic foot ulcers (Leong et al. 2018). Several reports from 2014 to 2021 show that Enterococcus species are involved in approximately 10% of total SSTIs, among the top SSTI-associated pathogens in all countries examined (Rajkumari et al. 2014, Heitkamp et al. 2018, Wilcox and Dryden 2021). Within the genus, *E. faecalis* and *E. faecium* are the most frequently isolated from SSTI (Jones et al. 2003, Rajkumari et al. 2014, Heitkamp et al. 2018). While *E. faecium* is more frequently vancomycin-resistant, *E. faecalis* are associated with a higher number of infections, accounting for ∼5% of all surgical site infections in the US and Europe (Giacometti et al. 2000, Lake et al. 2018). Diabetic lower limb infection is also highly associated with *E. faecalis* infection, found in 13.2–45.2% of cases and is the most frequently isolated bacterial species in lower limb bone infection in diabetics (Ge et al. 2002, Citron et al. 2007, Chellan et al. 2010, Jneid et al. 2018, Carro et al. 2020). *E. faecalis* is tolerant or resistant to a variety of antibiotics and also possesses multiple mechanisms to avoid immunosurveillance (Ruiz-Garbajosa et al. 2009, Kouidhi et al. 2011, Kao and Kline 2019). Together, these persistent features enable *E. faecalis* to cause infections ranging from the life-threatening like infective endocarditis and bloodstream infection (Caballero-Granado et al. 2001, Anderson et al. 2005, Luzzaro et al. 2011, Reigadas et al. 2013, Dahl et al. 2016) to sometimes chronic infections such as urinary tract infection (particularly catheter-associated UTI (CAUTI)) and wound infection (Ge et al. 2002, Gjodsbol et al. 2006, Citron et al. 2007, Foxman 2010, Rhoads et al. 2012, Wolcott et al. 2016, Gaston et al. 2021). A 2019 report estimated more than 30 000 of deaths worldwide could be attributed to antibiotic resistant *E. faecalis* (Antimicrobial Resistance 2022). Many *E. faecalis* infections, such as wound infection and CAUTI, are polymicrobial with commonly co-isolated species including *Staphylococcus* species, *Acinetobacter baumannii*, *Escherichia coli*, *Klebsiella* species, and *Proteus mirabilis* (Ronald 2003,

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Reyes et al. 2010, Gaston et al. 2021; Y. C. Zheng et al. 2019, Wang et al. 2020), depending on the infection site. While the majority (> 80%) of diabetic foot infections are polymicrobial in nature (Citron et al. 2007, Jneid et al. 2018), this trend is taken to the extreme with *E. faecalis*, with fewer than 1% of *E. faecalis*-involved infections being mono-species (Citron et al. 2007). Mixed-species infections are often correlated with poorer prognoses such as longer hospital stays, longer intensive care unit stays, higher incidence of septic shock, higher in-hospital mortality for bloodstream infections, failed treatments, higher mortality rates in CAUTI, and increased need for amputations for diabetic wound ulcers (Coombs et al. 2014, Pinholt et al. 2014, Shettigar et al. 2018, Falcone et al. 2019, Hitam et al. 2019, Zheng et al. 2019, Gaston et al. 2021). Given the poor outcomes associated with polymicrobial infections, coupled with the fact that antibiotic treatment is often less effective against multi-species communities (Short et al. 2014), there is an urgent need to consider alternative therapeutic strategies for common mixed-species infections. However, the pathogenic mechanisms of polymicrobial infections, compared to mono-species infections, are not well studied.

Neutrophils are one of the first responding leukocytes that infiltrate injured or infected tissues (Rousseau et al. 2016, Ley et al. 2018). Equipped with a wide-array of anti-microbial enzymes and proteins, neutrophils use three primary mechanisms to eliminate pathogens: phagocytosis, degranulation, and neutrophil extracellular trap (NET) formation (Rosales 2018, Burn et al. 2021). Historically, neutrophils were considered non-specific in their activities, but more recent findings show that neutrophils react to their microenvironments to provide more specific and efficient responses (Malech et al. 2014, Scherer et al. 2021). For example, signals including different pathogen-associated molecular patterns (PAMPs), cytokines, intercellular interactions, and even surface material can determine the specific responses triggered in neutrophils (Mantovani et al. 2011, Selders et al. 2017). Specific and regulated neutrophil responses prevent inappropriate or excessive neutrophil activation which can result in collateral damage to host tissues or precipitate autoimmune reactions (Wilgus et al. 2013, Papayannopoulos 2018, Daniel et al. 2019). Previously, using a mouse model of wound infection, we observed that *E. faecalis* undergo a period of acute replication up to one day post infection (dpi) followed by a decline in bacterial load over the next three days, and finally a period of persistent infection that extends until at least seven dpi (Chong et al. 2017). Consistent with their role as a first responder to infection, neutrophil infiltration is prominent during the first three days of *E. faecalis* wound infection, suggesting that these cells play a role in the initial reduction of bacterial burden. However, despite the presence of neutrophils during acute *E. faecalis* wound infection, they are not able to clear the infection and prevent persistence. Similarly, in a mouse model of *E. faecalis* CAUTI, neutrophils are also recruited within one dpi. While neutrophil infiltration correlates with reduced *E. faecalis* colony forming units (CFU) during CAUTI, neutrophil presence does not prevent *E. faecalis* colonization of the catheter (Guiton et al. 2013). Studies of the interactions between *E. faecalis* and neutrophils show that opsonization, either by complement or antibodies, enable neutrophils to recognize and eliminate *E. faecalis* (Arduino et al. 1994). More detailed characterization of neutrophil responses towards *E. faecalis* is lacking.

Staphylococcus aureus is often co-isolated with *E. faecalis*, particularly at wound sites (Han et al. 2009, Reyes et al. 2010, Heinze et al. 2019, Shrestha et al. 2021). Of concern, *E. faecalis* can transfer vancomycin-resistance-encoding genes to methicillin-resistant *S. aureus* during co-infection (Weigel et al. 2007, de Niederhausern et al. 2011); however, little is known about what other mechanisms may contribute to the prevalence and persistence of this pair of microbial species. We recently showed that heme cross-feeding from *S. aureus* activates *E. faecalis* aerobic respiration within mature biofilms to increase ATP production and promote *E. faecalis* growth (Ch'ng et al. 2022). While there is an increasing appreciation that most *E. faecalis* infections are polymicrobial in nature, and more studies are establishing the mechanisms by which *E. faecalis* contribute to mixed-species communities (Keogh et al. 2016, Gaston et al. 2020), investigation into how these *E. faecalis*-containing microbial communities interface with the immune system are limited (Tien et al. 2017).

In this study, we characterized *E. faecalis*-stimulated responses mediated by neutrophils and evaluated neutrophil bactericidal activity following co-infection with *E. faecalis* and *S. aureus in vitro* and *in vivo*. Our data demonstrate that neutrophils primarily utilize phagocytosis and intracellular reactive oxygen species (ROS) to clear *E. faecalis*. Notably, *E. faecalis* does not induce NETosis, and when it predominates in a mixed-infection, *E. faecalis* can reduce *S. aureus*-induced NETosis via suppression of histone citrullination. Similarly, when *E. faecalis* is the predominant species in polymicrobial wound infections, survival of *S. aureus* is promoted *in vitro*. In mixed-species wound infection where *E. faecalis* is the predominant species, the bacterial burden of *S. aureus* increases compared to single-species infection, indicating a similar immunomodulation may occurred *in vitro* and *in vivo*. A dose-dependent correlation between reduced NETosis and enhanced *S. aureus* survival identifies *E. faecalis* as a potential risk factor in *S. aureus*-involved polymicrobial infection by impairing neutrophil-driven immunity.

Material and methods

Bacterial strains and cultures

Staphylococcus aureus strain USA300LAC was grown in tryptic soy broth (TSB) and *Enterococcus faecalis* OG1RF was grown in brain heart infusion (BHI) medium. To prepare the bacterial inoculum, a single colony was inoculated into 5 ml of liquid broth and grown statically for 16–20 hours at 37°C. *S. aureus* cells were washed and adjusted in PBS to O.D.600 nm= 1.5 (5×10^8 CFU/ml) and *E. faecalis* to O.D.600 nm= 0.5 (3×10^8 CFU/ml) before dilution to the indicated CFU or multiplicity of infection (MOI). For CFU enumeration, serial dilutions were spotted onto TSB agar (1.5% agar) plates for *S. aureus* and BHI agar plates (1.5% agar) for *E. faecalis*. For mixed cultures of *S. aureus* and *E. faecalis*, MRSAII selective plate (BIO-RAD, #63757) were used for *S. aureus* and BHI plates containing 25 g/ml of rifampicin were used for *E. faecalis*.

Mouse neutrophil isolation

Bone marrow neutrophils were freshly isolated from C57BL/6 mice. Tibias and femurs of the hind limbs were collected, and the bone marrow was flushed using a 25 G needle with ice cold phosphate-buffered saline (PBS). To purify neutrophils from bone marrow cells, magnetic-activated cell sorting (MACS) was performed using a LS column (Miltenyi Biotec, # 130–042-401) and mouse neutrophil isolation kit (Biolegend, #480058) following the manufacturer's instructions. Briefly, the cells were suspended in sorting buffer and labeled with biotin-antibody cocktail for 15 minutes on ice. After washing and resuspension, cells were incubated with streptavidin-conjugated nanobeads for another 15 minutes on ice before addition to the LS column installed on a magnetic separator and the flowthrough was collected containing purified neutrophils. Neutrophil purity at 90% of total cells

was confirmed by flow cytometry. In general, 4–6 × 10^6 neutrophils were collected from one mouse. After isolation, neutrophils were rested at a cell density of 5 × 10^5/well for 24-well plates and 1×10/well for 96-well plates in Hank's balanced salt solution (HBSS) with calcium, magnesium (Thermofisher, #14025076), and addition 10% of mouse serum at 37°C for 30 to 60 minutes before further stimulation or infection.

In vitro neutrophil infection assay

This assay was modified from a previous study (Pilsczek et al. 2010). Briefly, 24-well or 96-well plates (Nunc, ThermoFisher) were coated with 10% fetal bovine serum (FBS) at 4°C for overnight. Bacteria used for infection were first grown overnight in BHI at 37°C as described above, washed with PBS, and resuspended in HBSS with 10% mouse serum, and incubated for 30 minutes on ice prior to washing and normalization in HBSS without serum to the required MOI. At the same time, neutrophils were isolated and rested as described above. Neutrophils were then infected with bacteria in the same volume of HBSS to achieve a final concentration of 5% mouse serum. The infections proceeded at 37°C until the desired timepoint as follows: 4 hour incubation for bacterial killing assays and bioimaging assays, 6 hours for ROS detection assays or other specified time points for flow cytometry. To measure the viable bacteria after incubation with neutrophils, triton-x was added (final concentration at 0.1%) to lyse neutrophils and release intracellular bacteria. For intracellular ROS assays, neutrophils were pre-incubated with diphenyleneiodonium chloride (DPI, Sigma-Aldrich #D2926-10MG) during the resting stage at 37°C for 30 minutes prior to washing (Buck et al. 2019). Cells were then resuspended in Hank's balance salt solution (HBSS) with 10% of mouse serum before inoculation with bacteria. For some experiments, indicated compounds such as 30 μM of cytochalasin B (abcam, #ab143482) and D (abcam, #ab143484), or 80 nM of phorbol myristate acetate(PMA) plus 1 μg/ml of ionomycin (Cell Activation Cocktail, Biolegend #423 302) were added to the culture medium.

Neutrophil cytotoxicity assay

For cytotoxicity assays, ATPlite 1step Luminescence Assay System (PerkinElmer, #6 016 736) was used to detect adenosine triphosphate (ATP) released upon cell death. Supernatants of cells with indicated treatments were collected and stored at -80°°C until detection. Samples were thawed and used in the assay according to the manufacturer's instructions. In short, 100 μl of the supernatants were added to 100 μl of the assay reagent in a white-well plate. The plate was incubated in the dark with shaking for 5 minutes at room temperature before luminescence detection by plate reader (Tecan Infinite 200 PRO spectrophotometer). For data analysis, the untreated group was set at 0% cell death while cells incubated with 0.1% triton-x were considered 100% cell death.

Immunofluorescence microscopy

For immunofluorescence imaging, neutrophils were seeded in either glass-bottom plates (ibidi, μ-plate) or 6-well plates (Nunc, ThermoFisher) with square cover slips inside. At specified times, neutrophils were fixed with 4% paraformaldehyde (Biolegend, #420801) for 10 minutes at room temperature. For staining of citrullinated histone H3, cells were permeabilized with 0.1% triton-x for 10 minutes in PBS, while this permeabilization step was skipped for the staining of externalized neutrophil elastase. After washing with PBS, the cells were incubated with 2% bovine serum albumin (BSA) in PBS for 1 hour at room temperature to block nonspecific antibody binding. Samples were then incubated overnight at 4°C with the primary antibody (neutrophil elastase, Abcam #ab68672; citrullinated histone H3, Abcam #ab5103; all were used at 1 : 500). On the second day, samples were washed and then incubated with secondary antibody (1:250) for 1 hour at room temperature in the dark. Finally, Hoechst 33342 (100 ng/ml) was used to stain DNA for 15 minutes at room temperature. Images were taken with a Carl Zeiss Axio Observer and cells in each field of view were manually counted. For each sample, at least 10 fields of view were imaged for each sample, and 100–200 cells were counted per sample.

DNA detection in cells with compromised cell integrity

To detect compromised cells, neutrophils were pre-stained with Hoechst 33342 (100 ng/ml) during resting for 45 minutes in the dark at 37°C after isolation from the bone marrow. Neutrophils were then washed and inoculated with bacteria in the presence of 500 nM of Sytox Orange (ThermoFisher #S11368) to stain DNA of the cells that have lost membrane integrity. After 4 hours of incubation at 37°C, cells were washed and fixed for microscopy. One image per sample per experiment was taken at 5x magnification and analyzed with ImageJ, by which the mean fluorescence intensity of the Sytox Orange signal was calculated.

Flow cytometry

A Fortessa X flow cytometer was used to evaluate the surface expression of degranulation markers and integrins. To stain the cells, approximately 8 × 10^5 neutrophils were collected after the indicated stimulation, washed with PBS, and stained with LIVE/DEAD Fixable Viability Dead Cell Stains (1 : 1000, reconstituted according to manufacturer's instructions) (ThermoFisher) for 30 minutes at 4°C in the dark. Cells were then washed with 2% FBS in PBS and incubated with Fc blockers (50 ng/ml) (Biolegend, TruStain FcX™ Antibody) for 15 minutes to avoid nonspecific binding. A cocktail of antibodies recognizing CD45 (Biolegend, clone 30-F11), Ly6G (Biolegend, clone 1A8), and CD11b (Biolegend, clone M1/70) were then added in the final dilution of 1:1000 and incubated with the neutrophils for 30 minutes at 4°C in the dark. Cells were washed once more with PBS prior to flow cytometry. Analysis of neutrophil viability was performed after gating on neutrophils (CD45+, Ly6G+, CD11b+) with FlowJo, version 10.

In vivo mouse wound infection

Mouse wound infections were performed as previously described (Chong et al. 2017). Briefly, bacteria were grown overnight and normalized in PBS to prepare the inoculum, which was confirmed for accuracy by CFU enumeration. Mice were first anesthetized with ketamine and xylazine. Hair on the dorsal skin was shaved and further removed with hair removal gel. After disinfecting with 70% ethanol, a single wound was created on the dorsal skin by 6-mm biopsy punch. 10 μl of the bacterial inoculum was added to the wound and left to air dry for 3 minutes. A piece of adhesive dressing (Tegaderm, 3 M) was applied to seal the wound. The entire procedure was performed under a heat lamp to prevent hypothermia. At 1 dpi, a 1×1 cm piece of the wound tissue (including the Tegaderm) was collected and homogenized in PBS. Homogenates were then diluted and CFU enumerated on selective media.

Statistical analysis

GraphPad Prism 9 software was used for statistical analysis. Analytic tests used for evaluating differences among groups with parametric distributions are indicated in each figure legend. Data

are presented with mean or mean ± SD of at least 3 biologically independent experiments. *P* values of < 0.05 were considered statistically significant.

Ethics statement

Animal procedures were reviewed and approved by Institutional Animal Care and Use Committee (IACUC) in Nanyang Technological University (AUP# A19061) and performed accordingly.

Results

E. faecalis infection triggers neutrophil phagocytosis and ROS production, but not NETosis

To begin to understand how neutrophils contain *E. faecalis* infection, we infected mouse neutrophils at a MOI of 10 and enumerated bacterial survival *in vitro*. After 4 hours of exposure to neutrophils, *E. faecalis* CFU were 80% lower compared to bacteria treated identically except for the presence of neutrophils (Fig. 1A). This reduction in bacterial CFU was abrogated when phagocytosis inhibitors cytochalasin B and D were added (Fig. 1A), indicating that neutrophil killing *of E. faecalis* follows phagocytosis, consistent with previous reports of opsonophagocytic killing of *E. faecalis* by neutrophils (Arduino et al. 1994) Upon phagocytosis, neutrophils produce ROS, which accumulates in the phagosome to achieve antimicrobial activity. To evaluate the importance of ROS in the control of *E. faecalis* infection, we pretreated the neutrophils with diphenyleneiodonium (DPI), a nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibitor, to prevent the production of intracellular ROS. Incubation of *E. faecalis* with DPI-pretreated neutrophils resulted in higher CFU recovery than vehicle (DMSO)-treated neutrophils (Fig. 1B), suggesting the production of ROS plays an important role in the elimination of intracellular *E. faecalis* following phagocytosis. Neutrophil viability was monitored in the presence of inhibitors and no significant cell death was detected (Fig. S1A), indicating that their reduced antimicrobial function was not due to cytotoxicity but represented suppressed antibacterial activity.

In addition to intracellular killing by phagocytosis, neutrophils are also capable of killing extracellular bacteria via NETosis and degranulation. One of the hallmarks of NETosis is decondensation of chromatin prior to its release into the extracellular environment. Chromatin decondensation is mediated by peptidylarginine deiminase 4 (PAD4) that loosens chromatin via histone citrullination (Y. Wang et al. 2009). We evaluated both the morphology of nucleus and histone citrullination in neutrophils by immunofluorescence after 4 hours of incubation (Fig. 1C). When infected with *E. faecalis*, neutrophil nuclear morphology was largely unchanged, compared to neutrophils stimulated with NETosis activators (Brinkmann et al. 2004) phorbol myristate acetate(PMA) and ionomycin (P+I) resulting approximately 40% of the total cells exhibiting decondensed chromatin (Fig. 1C, D). Similarly, fewer than 10% of neutrophils infected with *E. faecalis* were positive with citrullinated histones H3 (H3Cit), comparable to the untreated group, whereas P+I treatment again resulted in around 40% of neutrophils with histone citrullination (Fig. 1C, E). Collectively, these results indicate that *E. faecalis* does not induce PAD4-dependent NETosis.

Neutrophils are equipped with multiple types of antimicrobial granules, including azurophilic granules, specific granules, and secretory vesicles. Neutrophils release granules upon different microbial or host signals, such as lipopolysaccharide (LPS) or IL-8 (Lacy 2006, Naegelen et al. 2015). By measuring granule markers on the surface of neutrophils, which indicate fusion of the granule membrane to the cell membrane and release of the granular content, we established the degranulation profile upon *E. faecalis* infection. Azurophilic granules, also known as primary granules, contain myeloperoxidase (MPO) and neutrophil elastase (NE), and is recognized by CD63 (Naegelen et al. 2015). Upon *E. faecalis* infection, neutrophil surface levels of CD63 increased significantly (Fig. 2A), indicating the triggering and release of azurophilic granules. Surface levels of CD15, a marker for secondary granules, were also increased following *E. faecalis* infection (Fig. 2B). By contrast, *E. faecalis* infection resulted in a slight increase in secretory vesicle marker CD14 and a reduction of secretory vesicle marker CD16 on the neutrophil surface (Fig. 2C and D), suggesting that the degranulation of secretory vesicles is not stimulated. The reduction of CD16 could be a result of phagocytosis since CD16 also acts as a receptor for antibodies that neutrophils use to recognize and engulf the labeled *E. faecalis*, internalizing CD16 during the process (Webster et al. 2006).

Altogether, these data show that the neutrophil response to *E. faecalis* infection consists primarily of phagocytosis and ROS-mediated killing. *E. faecalis* can also trigger extracellular antimicrobial mechanisms including azurophilic degranulation. However, degranulation of secretory vesicles and NETosis were not induced by *E. faecalis*, suggesting that intracellular antimicrobial mechanisms play the major role in controlling *E. faecalis* infection.

S. aureus CFU increases with co-infection of *E. faecalis* in a dose-dependent manner

Since *E. faecalis* is often co-isolated with *S. aureus*, we investigated whether the mixed-species infection with *E. faecalis* would affect the killing of either bacterial species by neutrophils. Similar to *E. faecalis*, the inhibition of phagocytosis also reduced the neutrophil-mediated killing of *S. aureus* (Miller et al. 2019). However, even pre-treated with cytochalasin, neutrophils can still perform bactericidal activity to control *S. aureus* burden through NETosis (Brinkmann et al. 2004). *S. aureus* promotes NETosis (Malachowa et al. 2013), which is partially inhibited by DPI treatment, and NETosis is the major antimicrobial mechanism used by neutrophils to control *S. aureus* infection (Pilsczek et al. 2010), so we hypothesized that co-infection with *E. faecalis* could inhibit NET formation and in turn protect *S. aureus*. We first infected neutrophils with different ratios of *E. faecalis* and *S. aureus*, ranging from 1:10 (Fig. 3A), 1:1 (Fig. 3B), to 10:1 (Fig. 3C). Co-culture of *E. faecalis* with *S. aureus* in any ratio in the absence of neutrophils did not lead to significant increase of either species (Fig. S2). In fact, co-culture of *E. faecalis* slightly reduced *S. aureus* CFU at ratio of 1:1 (Fig. S2B) and 10:1 (Fig. S2C). By contrast, when neutrophils were coinfected at a low inoculum (MOI 1) of *E. faecalis*, the CFU of co-infected *S. aureus* (MOI 10) in the mixed-species infection was comparable to single-species *S. aureus* infection at MOI 10 (Fig. 3A). When an equivalent MOI of each species (MOI 10) was used to infect neutrophils, *S. aureus* CFU increased slightly compared to single-species infection (Fig. 3B), and the increase was more pronounced when even fewer *S. aureus* (MOI 1) were mixed with more (MOI 10) *E. faecalis* (Fig. 3C). The fold change of *S. aureus* CFU from mixed-species infection to single-species infections showed an *E. faecalis* dose-dependent increase (Fig. 3D), indicating that *E. faecalis* may protect *S. aureus* from neutrophil killing. Since we hypothesized that *E. faecalis* co-infection protects *S. aureus* by inhibiting NET formation, which is a form of programmed cell death, we examined the level of neutrophil cell death in each infection condi-

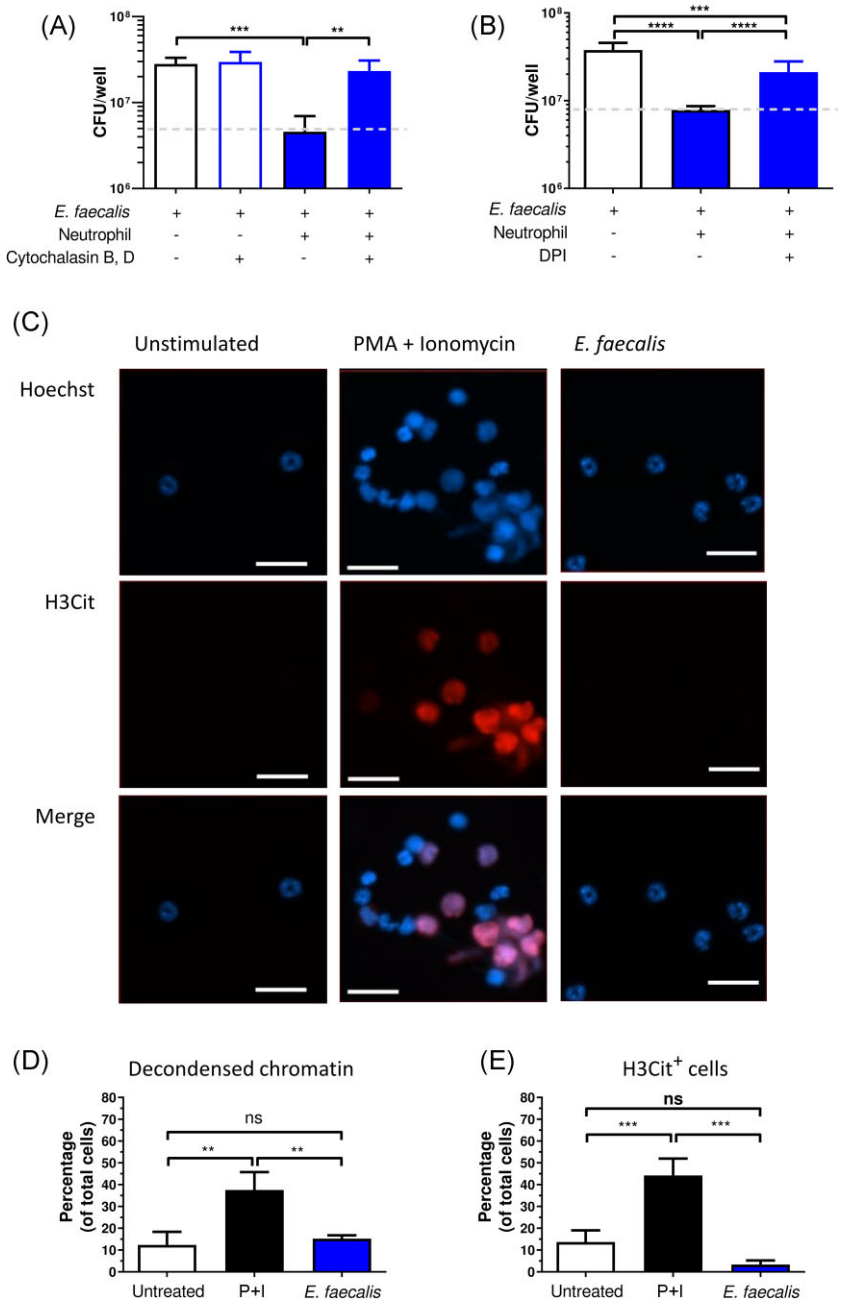

Figure 1. Neutrophils control *E. faecalis* infection through phagocytosis and intracellular ROS production, with no induction of NETosis. (A-B) Bacterial CFU after incubation with neutrophils for four hours, with the addition of compounds where indicated. (A) *E. faecalis* infection with neutrophils for 4 hours in the presence or absence of cytochalasin B and D (30 μM each). (B) Neutrophils pretreated with 20 μM of diphenyleneiodonium chloride (DPI) for 30 minutes before infection with *E. faecalis* for 4 hours. (C) NETosis of neutrophils was examined by immunolabeling after 4 hours of treatment with PMA + ionomycin or *E. faecalis*. Decondensed chromatin (Hoechst, blue) and presence of citrullinated histone H3 (red) were determined (scale bar = 20 μm). Representative images from 3 independent experiments with 6 biological replicates are shown. (D-E) Neutrophil chromatin decondensation and histone H3 citrullination assessed after 4 hours incubation with indicated treatments was measured from 3 independent experiments and total 3–6 biological replicates. P+I indicates co-treatment with PMA and ionomycin. The percentage of (D) cells with decondensed chromatin and (E) cells positive with citrullinated histone H3 were evaluated by examining over 100 cells per experiment based on microscopy. All analyses are presented as mean ± SD, and dotted lines (A, B) indicate bacterial inoculum. One-way ANOVA with Tuckey for post-hoc test (A, B, D, E) was used for statistical analyses. **= $P < 0.01$, *** = $P < 0.001$.

tion using the ATPlite 1step Luminescence Assay System and expected to observe reduced cytotoxicity in mixed-species infection compared to *S. aureus* single-species infection. *E. faecalis* infection alone did not induce cytotoxicity, while *S. aureus* single species infection resulted in significant cell death (Fig. S1B) consistent with other reports (Pilsczek et al. 2010). In mixed species infection, the viability of neutrophils was partially restored compared to that of *S. aureus* single-species infection (Fig. S1B). This result was further confirmed with Sytox Orange staining, which only labels cells that have lost membrane integrity. While *S. aureus* infection resulted in high intensity Sytox Orange signal in neutrophils, the intensity was reduced with co-infection of *E. faecalis* (Fig. S1C and D). Together, these results align with our hypothesis that *S. aureus*-induced NETosis may be suppressed during co-infection.

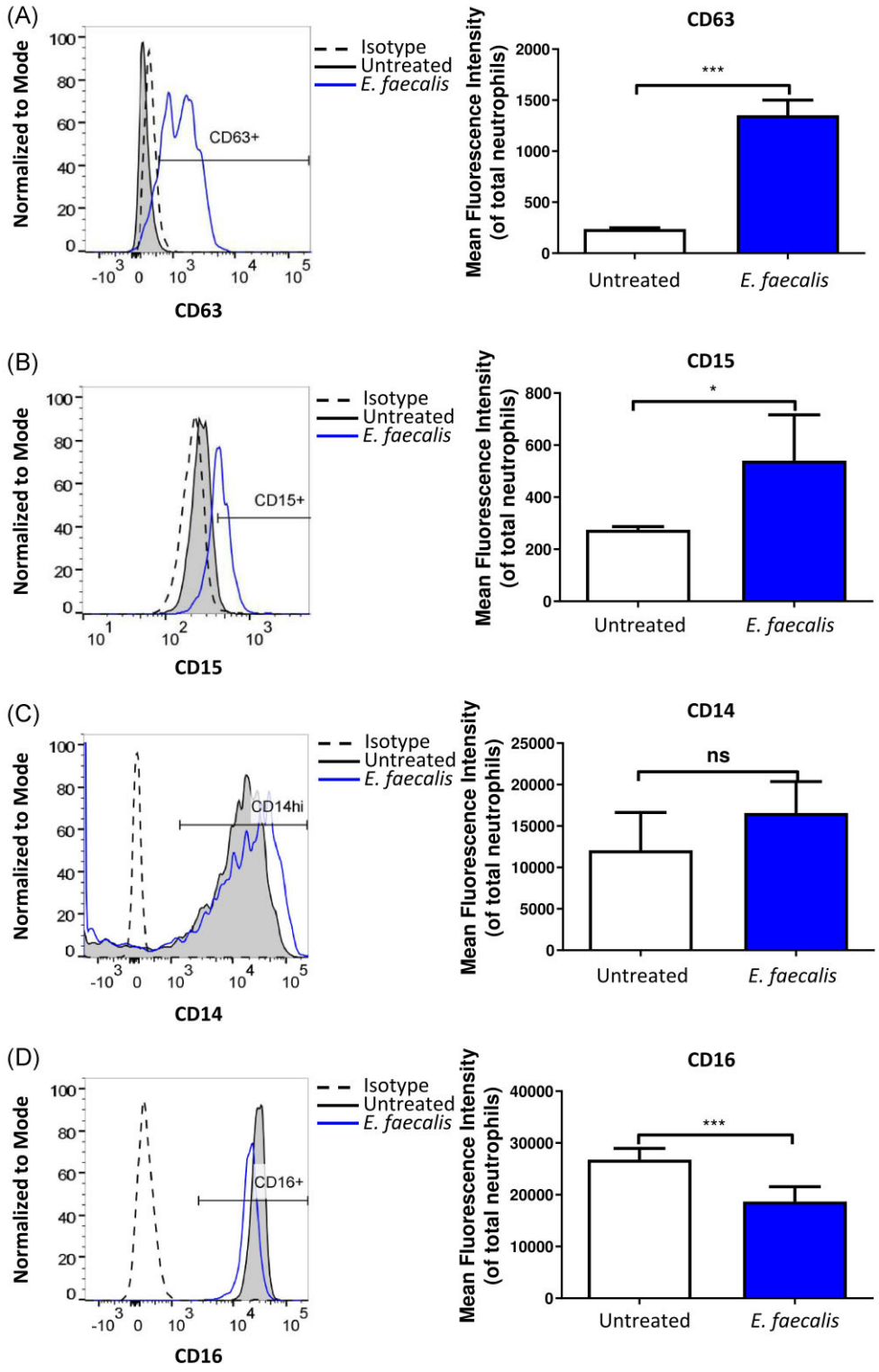

Figure 2. *E. faecalis* induces degranulation of azurophilic granules and specific granules, but not secretory vesicles. (A-D) Degranulation of neutrophils was evaluated by surface level of granule markers. Surface levels of (A) CD63, (B) CD15, (C) CD14, and (D) CD16 on neutrophils (gated on CD45+, Ly6G+) were analyzed by flow cytometry after 4 hours with or without *E. faecalis* infection. Histogram (left) and mean fluorescent intensity (right) of each marker are shown. For mean fluorescence intensity, bars represent means±SD. Data collected from 3 to 5 biological replicates, performed in 3 independent experiments, were pooled and analyzed with One-way ANOVA. * = $P < 0.05$, *** = $P < 0.001$.

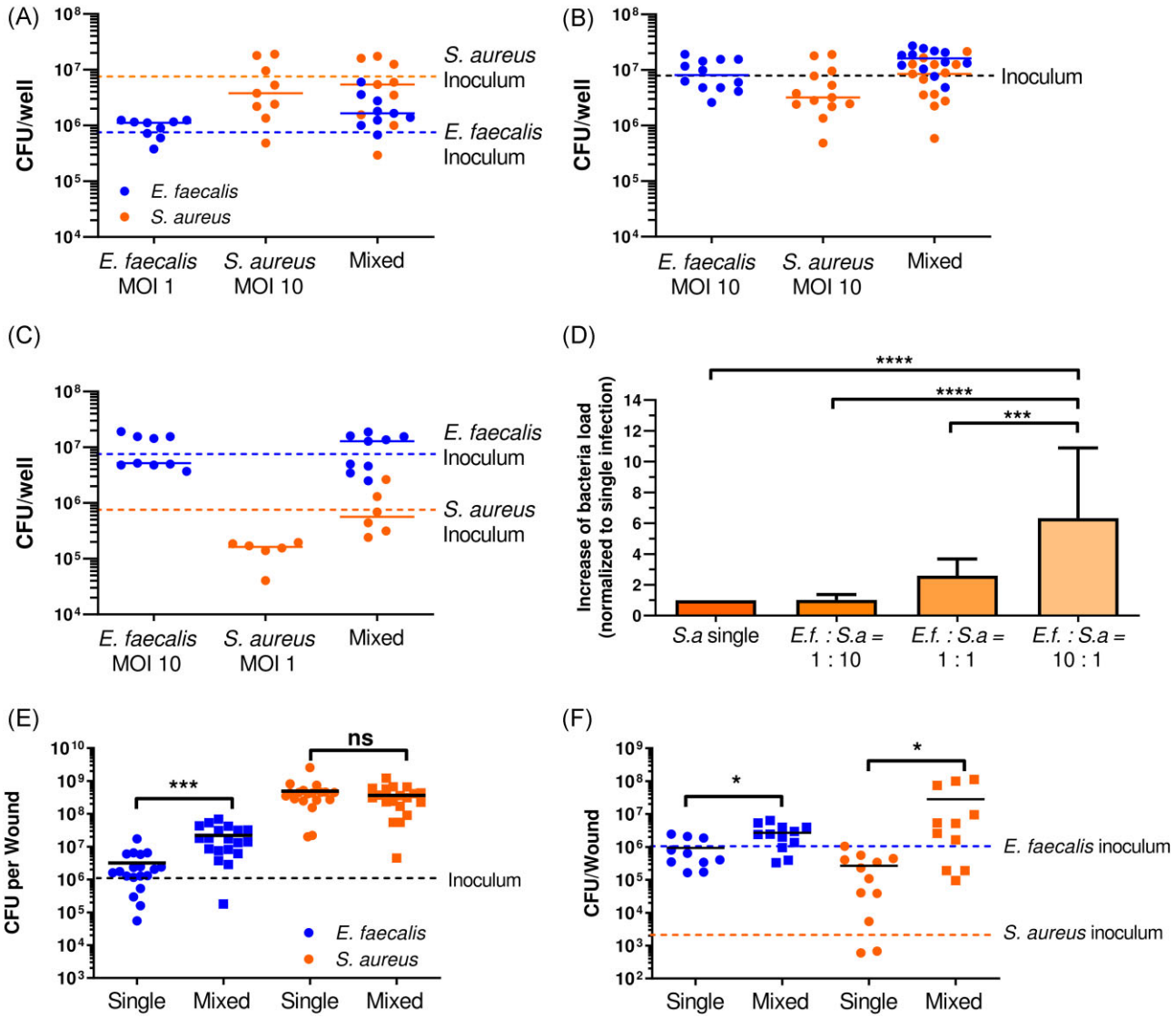

Figure 3. Co-infection promotes *S. aureus* survival both *in vitro* and *in vivo* in an *E. faecalis* dose-dependent manner. (A-D) *In vitro* bacterial load of *E. faecalis* and *S. aureus* after 4 hour incubation with neutrophils. (A-C) CFU of *E. faecalis* and *S. aureus*, from single- or mixed-species infection at the indicated inoculum were enumerated. Each condition was examined in 3–5 independent experiments with 6–13 biological replicates. Horizontal bars represent CFU means and dotted lines to indicate bacterial inoculum. (D) Fold change of *S. aureus* bacterial load was measured by normalizing the CFU of each co-infection to its cognate single-species infection. Bars represent means±SD for fold change relative to each single-species infection. Statistics were performed with one-way ANOVA and differences were considered significant for * $P<0.05$, *** $P<0.001$. ns = not significant. (E-F) *In vivo* CFU recovered from mouse wounds 24 hours post infection. (E) Mouse wounds were infected with *E. faecalis* (10^6), *S. aureus* (10^6), or a 1:1 mixture. Three independent experiments were conducted with total of 19 mice. (F) Mouse wounds were infected with *E. faecalis* (10^6), *S. aureus* (2×10^3), or mixed. Three independent experiments were conducted with3-4 mice each experiment. Horizontal bars represent CFU means and dotted lines to indicate bacterial inoculum. Differences between mixed- and single-species infection were analyzed by unpaired t-test within each species and differences were considered significant for * $P < 0.05$, *** $P<0.001$, **** $P<0.0001$. ns = not significant.

To determine whether the MOI-dependent enhanced bacterial CFU in mixed-species infection observed *in vitro* also occurs *in vivo*, we used a mouse wound infection model, introducing *E. faecalis* and *S. aureus* at different inoculum ratios and enumerating CFU in the wound tissue 24 hours post infection (hpi). This model was chosen because neutrophil infiltration is observed within 24 hpi, providing a reasonable approximation for the *in vitro* infection experiments (Chong et al. 2017). Following inoculation of the wound with a 1:1 ratio of *E. faecalis* and *S. aureus* (10^6 CFU of each), similar to the *in vitro* experiments, *E. faecalis* CFU were approximately 7-fold greater compared to the single-species infection (Fig. 3E). By contrast, *S. aureus* replicated to higher numbers than *E. faecalis* and CFU were not changed in the mixed-species infection (Fig. 3E). To determine whether reducing *S. aureus* in the input ratio would favor *S. aureus* growth *in vivo*, as we observed for 1:10 *E. faecalis*: *S. aureus* co-infection *in vitro*, we inoculated wounds with a reduced *S. aureus* inoculum while keeping the *E. faecalis* inoculum at 10^6 CFU. This mixed-ratio infection resulted in significantly greater CFU for both microbes compared to single-species infection, with *E. faecalis* CFU increased slightly (less than 3-fold), and *S. aureus* increased by more than 100-fold (Fig. 3F). These results correlated with *in vitro* observations where the higher inoculum of *E. faecalis* protects *S. aureus* from neutrophil-mediated antimicrobial functions during co-infection.

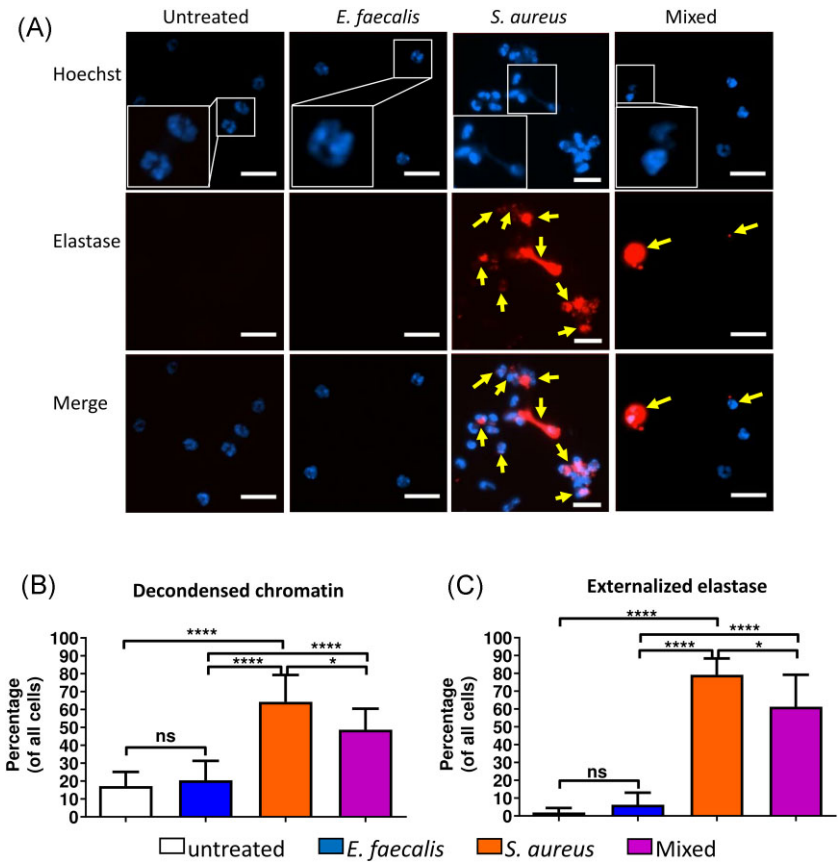

Figure 4. *E. faecalis* suppresses *S. aureus*-induced NETosis. Evaluation of NETosis following single- and mixed-species infection after 4 hours of incubation. (A-C) Neutrophils infected with MOI 10 of *E. faecalis* and/or *S. aureus*. (A) NETosis was detected by neutrophil elastase-associated externalized DNA. Representative images of neutrophil DNA stained with Hoechst (blue) and immunofluorescence for neutrophil elastase (red) (scale bar = 20 μm). The percentage of (B) cells with decondensed chromatin and (C) cells with extruded neutrophil elastase were evaluated by examining over 100 cells per experiment. All quantified results collected from 3 independent experiments with 5–10 biological replicates are presented with means±SD. Statistical analysis was performed with one-way ANOVA using Tukey for post-hoc test and differences were considered significant for * $P < 0.05$, **** $P<0.0001$. ns = not significant.

E. faecalis co-infection reduces NETosis induced by *S. aureus*

NETosis is an established neutrophil-mediated defensive mechanism against *S. aureus* infection. Given that *E. faecalis* did not induce NETosis at all (Fig. 1C-E), along with the observation that co-infection reduced the level of neutrophil death compared to *S. aureus* single-species infection (Fig. S1B–D), we hypothesized that *E. faecalis* may suppress *S. aureus*-induced NETosis, leading to increased *S. aureus* CFU. To address this hypothesis, we use *in vitro* assays to evaluate the morphology of neutrophil nuclei (Fig. 4A) and formation of NETs as indicated by externalized neutrophil elastase associated with DNA (Fig. 4A), as well as the overall amount of extracellular DNA (Fig. 4B). At MOI 10, *S. aureus* infection induced a large percentage of neutrophils to undergo NETosis, resulted in significant increase in decondensed chromatin (Fig. 4B) and neutrophil elastase-associated externalized DNA (Fig. 4C) compared to uninfected neutrophils. While *E. faecalis* alone at MOI 10 didn't trigger any of the NETosis-related markers, the addition of *E. faecalis* in mixed-species infection significantly reduced the level of *S. aureus*-induced NETosis compared to *S. aureus* single infection (Fig. 4B, C). This reduction was further evaluated by examining the number of cells that exhibited decondensed chromatin at different inoculation ratios of *E. faecalis* : *S. aureus* of 1:10 and 10:1. We observed a dose-dependent effect of *E. faecalis* on nuclear morphology that correlates with the bacterial load (Fig. 3A–D), where the low inoculum (MOI 1) of *E. faecalis* failed to reduce NETosis induced by MOI 10 of *S. aureus* (Fig. S3A and B), while *E. faecalis* at MOI 10 significantly attenuated the NETosis induced by a low inoculum (MOI 1) of *S. aureus* (Fig. S3C and D).

To explore the potential mechanism behind reduced NETosis, we next evaluated *in vitro* histone citrullination to investigate whether the PAD4-mediated NETosis pathway was attenuated by co-infection of *E. faecalis*. Single-species infection with *S. aureus* induced histone H3 citrullination in approximately 36.22% (±7.11%) of the neutrophils, significantly higher than uninfected neutrophils (7.57±5.62%) (Fig. 5A–B). Similar to other NETosis features, *E. faecalis* alone did not induce histone H3 citrullination (4.80±2.38%), and the co-infection with S. aureus attenuated citrullination (25.19±5.62%) compared to *S. aureus* single-species infection (Fig. 5A–B). Visual inspection revealed that *S. aureus* infected neutrophils formed aggregated NET structures that associated with extracellular *S. aureus* (Fig. S4A). In mixed-species infection, however, not only was the distribution of NET structures much lower than observed for *S. aureus* single-species infection, but some neutrophils associated with *S. aureus* also failed to undergo NETosis (Fig. S4B). Collectively, these results show that *E. faecalis* can suppress *S. aureus*-induced NETosis by attenuating histone citrullination and neutrophil-mediated antimicrobial functions, which lead to enhanced *S. aureus* bacterial survival following coinfection.

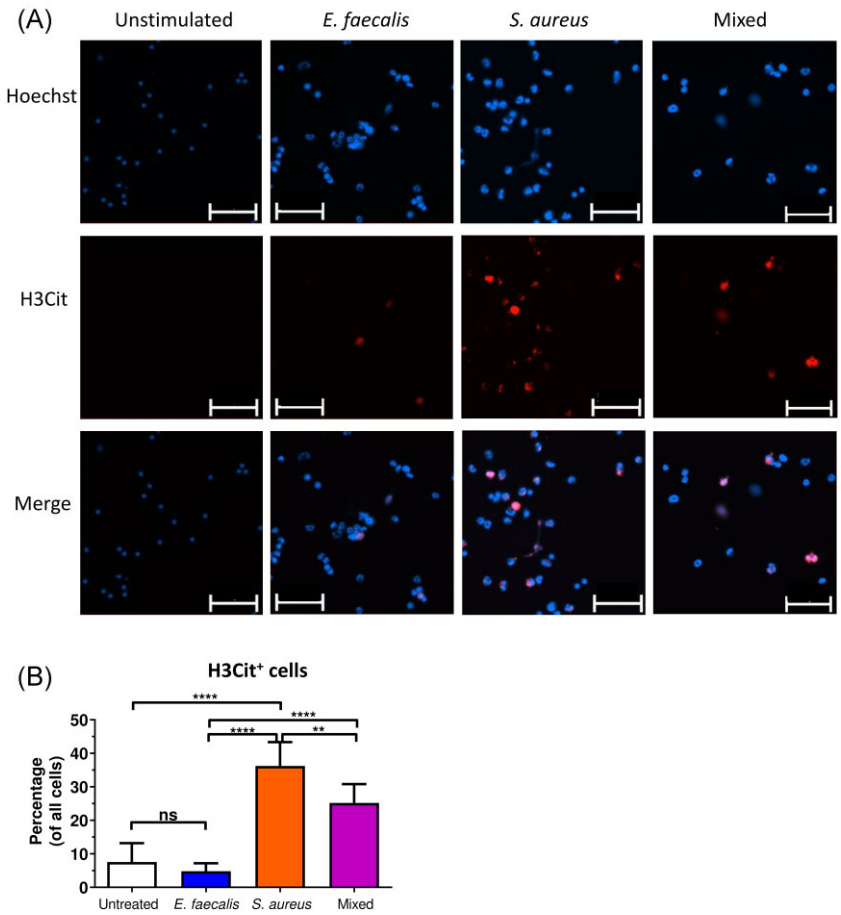

Figure 5. *E. faecalis* co-infection attenuates *S. aureus*-induced citrullination of histone H3. (A) Histone citrullination detected by immunofluorescent staining of citrullinated histone H3 (red) with all cells stained with Hoechst for DNA (blue). Representative images are shown. Scale bar = 50 μm. (B) Percentage of cells with citrullinated histone H3 evaluated by examining over 100 cells per experiment. Quantified results collected from 3 independent experiments with 7–9 biological replicates are presented with means±SD. Statistical analyses were performed using one-way ANOVA with Tukey for post-hoc test and differences were considered significant for *** $P < 0.001$. ns = not significant.

Discussion

Polymicrobial infections often occur in chronic infections such as cystic fibrosis and diabetic wounds and are serious medical challenges that complicate treatment regimens as well as increase the risk of antibiotic resistance (Shettigar et al. 2018, Heinze et al. 2019, Hitam et al. 2019, Zheng et al. 2019). Microbial communities use various mechanisms to promote their survival, including metabolite exchange as energy sources for other microbes (Keogh et al. 2016, Rakoff-Nahoum et al. 2016, Ch'ng et al. 2022), establishing biofilms which promote persistence in the face of immune and antibiotic activities (Pastar et al. 2013, Klein et al. 2018), and induction or transfer of virulence factors among community members (de Niederhausern et al. 2011, Tay et al. 2016). In this study, we examined how polymicrobial infection can influence the nature of the host neutrophil response. We found that *E. faecalis* alone not only fails to induce NETosis, but it can also actively suppress NET formation induced by *S. aureus*. Moreover, coinfection of neutrophils with both *E. faecalis* and *S. aureus* promotes the survival of *S. aureus in vitro* in an *E. faecalis* dose-dependent manner. We observed a similar outcome in a mouse model of wound infection, in which the bacterial load of *S. aureus* increased when *E. faecalis* was the predominant species in the mixed-species infection.

Despite consistent results *in vitro* and *in vivo*, in which coinfection with *E. faecalis* results in greater *S. aureus* recovery, it is unclear whether the increased *S. aureus* population *in vivo* is a result of impaired NETosis, as we observe *in vitro*. In addition to neutrophil-specific responses, other host cells (e.g. platelets, fibroblasts, and epidermal cells) or bacterial metabolite exchange may also contribute to the enhanced colonization in the *in vivo* environment, and further investigation is underway to understand the spectrum of interactions that occur between this polymicrobial community and the host.

While opsonization is required for efficient neutrophil killing of *E. faecalis* (Arduino et al. 1994, Gaglani et al. 1997), the detailed mechanism by which neutrophils kill this species had not been previously reported. Here we show that *E. faecalis* stimulates neutrophils to produce intracellular ROS that leads to *E. faecalis* clearance. While a mechanistic link between intracellular ROS and NETosis remain inconclusive, it has been suggested that intracellular ROS is linked to NETosis induction via activation of autophagy signaling pathways or via release of NE and MPO from primary granules into the cytoplasm (Stoiber et al. 2015). However, our data show that *E. faecalis*-induced ROS does not lead to NETosis. This uncoupling of ROS and NETosis is not unprecedented, given that neutrophils stimulated with GM-CSF and LPS also produce intracellular ROS that does not coincide with NET formation (Mol et al. 2021). One potential explanation is that *E. faecalis* drives neutrophils to primarily undergo phagocytosis, which would concentrate NADPH-ROS to the phagosomes as well as recruit primary

granules to fuse with phagosomes, providing antimicrobial enzymes to kill the engulfed pathogens (Nordenfelt and Tapper 2011, Hurst 2012). Together, this effector accumulation at the phagosomes could result in sequestration of NETosis activators, preventing NE and MPO from entering the cell nucleus and modifying histones for chromatin decondensation (Branzk et al. 2014), eventually leading to the inhibition of *S. aureus*-induced NETosis in mixed-infection. The sequestration of NE and MPO could be further enforced by *E. faecalis* during co-infection given that *E. faecalis* infection induces the degranulation of azurophilic granules, which is the primary storage site for NE and MPO, into extracellular environments.

Consistent with reduced NET formation following *E. faecalis* and *S. aureus* mixed-species infection, the level of citrullinated histones is also decreased in the mixed-species infection, indicating that co-infection of *E. faecalis* interferes with this pathway of NETosis activation and leads to the inhibition of NETosis. However, it is unclear whether *E. faecalis* can directly inhibit the activity of PAD4 to interfere with histone citrullination, or whether *E. faecalis* reduces NETosis by indirect mechanisms such as ROS sequestration. Interestingly, while *E. faecalis* alone fails to induce NETosis, and co-infection of *E. faecalis* reduces the levels of *S. aureus*-mediated NET production, a significant amount of NETosis still occurs in mixed-species infections, especially when *S. aureus* is in greater abundance. However, in this situation *E. faecalis* does not appear to be susceptible to NETosis-mediated killing since *E. faecalis* proliferates during mix-species infections. Microbial virulence factors such as capsule and endonuclease help species of *Streptococci* and *Staphylococci* to avoid NET killing activity or degrade NET structures, respectively (Papayannopoulos 2018, Bhattacharya et al. 2020). The *E. faecalis* strain used in these studies (OG1RF) does not encode capsule or any predicted secreted nucleases, so it remains to be determined how *E. faecalis* escapes NET-mediated inhibition.

In conclusion, we have demonstrated that neutrophils undergo distinct responses toward *E. faecalis* and *S. aureus*. Co-infection with *E. faecalis* and *S. aureus* skewed neutrophil responses away from NET formation in a dose-dependent manner, resulting in enhanced survival of NET-susceptible *S. aureus*. Immunotherapies of the future that are designed to promote immune clearance of pathogens must factor in this complexity given that infections are often polymicrobial in nature.

Author contributions

Patrick Hsien-Neng Kao, Jun-Hong Ch'ng, Kelvin K.L. Chong, Claudia J. Stocks, Siu Ling Wong, and Kimberly A. Kline

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Supplementary data

Supplementary data is available at *FEMSMC Journal* online.

Conflict of interest: The authors declare no conflicts of interest.

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