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Original Article

Porphyromonas gingivalis Outer Membrane Vesicles Enhance Aggregation and Phagocytic Clearance of Staphylococcus Aureus

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ABSTRACT

Staphylococcus aureus is a Gram-positive opportunistic pathogen responsible for diverse infections, including bacteremia (SAB). Patients with rheumatoid arthritis (RA) appear to be more prone to SAB, often experiencing more severe disease and complications such as infections in bones and joints. Meanwhile, Porphyromonas gingivalis, a Gram-negative oral pathogen, has been linked to RA development, largely due to its unique peptidylarginine deiminase (PPAD) enzyme and its highly active proteases called gingipains. These virulence factors are abundant in P. gingivalis outer membrane vesicles (OMVs), small structures released from the bacterial outer membrane. Our study reveals that these OMVs trigger S. aureus to form aggregates, a process dependent on both gingipains and PPAD, which can be reversed under certain conditions. Importantly, we found that exposure to P. gingivalis OMVs enhances the uptake of S. aureus by human neutrophils without causing noticeable neutrophil death. These observations suggest a potential mechanism by which P. gingivalis may suppress competing bacteria, promoting aggregation and neutrophil-mediated internalization of S. aureus. We hypothesize that this interaction could influence host-pathogen dynamics, as neutrophils carrying internalized bacteria might facilitate bloodstream dissemination, providing a possible explanation for the increased susceptibility of RA patients to SAB.

Keywords: PPAD, gingipains, Staphylococcus aureus bacteremia, Porphyromonas gingivalis, Outer membrane vesicles, Staphylococcus aureus, Rheumatoid arthritis

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Introduction

Staphylococcus aureus is a Gram-positive facultative anaerobe and opportunistic pathogen commonly present in humans and animals. Its primary colonization sites include the nasopharynx, skin, and gastrointestinal tract, with nasal carriage observed in up to 30% of the human population [1]. Accounting for colonization, environmental exposure, and transient carriage, it is estimated that as much as 60% of the world's population encounters this bacterium, establishing it as a significant member of the human microbiome [2-4]. Although colonization is typically asymptomatic, the bacterium's ability to inhabit multiple body sites allows it to cause a spectrum of diseases, ranging from superficial skin infections to implant-associated biofilm infections, septic arthritis, bacteremia, and endocarditis [5]. Moreover, S. aureus is a leading contributor to hospital-acquired infections, particularly affecting immunocompromised individuals. Its role in antimicrobial resistance (AMR) is also considerable, with multidrug-resistant strains accounting for roughly 25% of the AMR burden in high-income countries [6]. Among S. aureus infections, bacteremia (SAB) is particularly severe, with an incidence of 20–50 cases per 100,000 people and a 30-day mortality rate around 30%, often complicated by secondary infections [7, 8]. Notably, autoimmune disorders such as rheumatoid arthritis (RA) have been identified as risk factors for SAB [9, 10].

Although the mechanisms behind RA remain incompletely understood, the oral pathogen Porphyromonas gingivalis has emerged as a key contributor to disease onset [11]. This bacterium produces a unique enzyme, peptidylarginine deiminase (PPAD), which citrullinates host proteins and may trigger an autoimmune response in genetically predisposed individuals [11]. Such protein modifications can promote the formation of anti-citrullinated protein antibodies (ACPAs), which are highly specific to RA [12, 13]. PPAD-mediated citrullination is strongly influenced by P. gingivalis proteases called gingipains (RgpA/B and Kgp), which generate specific cleavage sites on host proteins; for example, cleavage by RgpA/B exposes a carboxyl-terminal arginine, the preferred substrate for PPAD [11, 14, 15]. Consequently, substantial research has focused on understanding the activity of gingipains and PPAD and their mechanisms of secretion.

One major secretion pathway involves packaging these virulence factors into outer membrane vesicles (OMVs), membrane-derived nanostructures that facilitate targeted delivery to host cells [16]. Beyond interacting with host tissues, OMVs may also affect other members of the microbiome. Notably, P. gingivalis OMVs have been shown to induce aggregation of S. aureus cells, likely reflecting interactions that occur in shared ecological niches within the oral cavity and nasopharynx [17–21]. Considering that RA is associated with an increased risk of SAB and that S. aureus is frequently found in patients with periodontitis [22–24], we sought to further investigate the mechanisms and consequences of OMV-induced staphylococcal aggregation.

Materials and Methods

Bacterial cultures

The cultivation of Porphyromonas gingivalis W83 and its PPAD knockout mutant [25] followed the procedure described by Stobernack *et al*. [24]. Initially, bacteria were spread onto Blood Agar No. 2 (BA2) and incubated for five days at 37°C under strictly anaerobic conditions (10% CO_2, 10% H_2, 80% N_2). Resulting colonies were transferred to enriched Brain-Heart Infusion (BHI) broth supplemented with L-cysteine (0.5 mg/mL), hemin (0.5 mg/mL), and menadione (10 µg/mL) and grown anaerobically at 37°C for 20 hours. Cultures were monitored for growth, and OD_{600} readings were taken upon reaching stationary phase.

Staphylococcus aureus HG001 carrying the pJL-sar-GFP plasmid for GFP expression [26] was maintained in Tryptic Soy Broth (TSB) containing 10 µg/mL erythromycin (Sigma Aldrich, Burlington, MA, USA). Overnight cultures grown at 37°C with agitation at 220 rpm were diluted into RPMI 1640 medium supplemented with 2 mM L-glutamine to an OD600 of 0.05 and incubated under shaking conditions at 37°C until reaching mid-exponential phase (OD_{600} ~0.5).

For cytotoxicity assays, the clinical Aggregatibacter actinomycetemcomitans isolate 30R [27] served as a positive control. Bacteria were initially grown on BHI agar enriched with 5% L-cysteine, 5 mg/L hemin, and 1 mg/L menadione at 37°C in 5% CO_2. Selected colonies were inoculated into BHI broth and incubated without shaking overnight until mid-logarithmic growth (OD_{600} ~0.5) was achieved.

P. gingivalis OMV isolation, quantification and purification

To isolate P. gingivalis outer membrane vesicles (OMVs), bacterial cultures in early stationary phase were first cleared of cells by centrifugation at 8,000 × g for 15 minutes at 4°C. The supernatant was then passed through a 0.22 µm filter (GE Healthcare Life Sciences, Chicago, IL, USA) to remove residual debris, followed by ultracentrifugation at 100,000 × g overnight at 4°C using an Optima™ XL-80K (Beckman Coulter Inc., Brea, CA, USA). The resulting OMV pellet was resuspended in 4 mL PBS and subjected to a second ultracentrifugation at 100,000 × g for 2 hours at 4°C to further purify vesicles.

For density gradient separation, OMVs were resuspended in 400 µL of 50% iodixanol (OptiPrep™ Density Gradient Medium; Sigma-Aldrich, Burlington, MA, USA) prepared in Buffer A (60 mM HEPES-NaOH, 0.85% w/v NaCl, pH 7.4). Subsequent gradient layers of 40%, 30%, 20%, and 10% iodixanol were prepared using Buffer B (0.85% w/v NaCl, 10 mM HEPES-NaOH) as diluent and carefully layered over the OMV-containing fraction, with the 10% layer on top. The gradient was centrifuged at 100,000 × g for 2 hours at 4°C. Fractions corresponding to 20% and 30% iodixanol, which were enriched in OMVs, were collected and pooled.

To verify OMV localization, proteins from each fraction were precipitated using trichloroacetic acid (TCA) and analyzed by Western blot with an anti-Omp41 antibody (GP2451) [28]. OptiPrep was removed by diluting the pooled OMV fractions in 4 mL Buffer B and performing overnight ultracentrifugation at 100,000 × g, 4°C. The OMV pellet was washed once more by resuspension in PBS followed by 2-hour ultracentrifugation, then finally resuspended in PBS and sterilized through a 0.22 µm filter. Prepared OMVs were stored at −80°C until further use.

Protein content of OMVs was quantified using the Pierce™ BCA Protein Assay Kit (Thermo Fisher,

Waltham, MA, USA). To ensure complete solubilization for accurate measurement, OMVs were treated with 2% SDS as previously described [29].

Human neutrophils

Primary human neutrophils were isolated from freshly collected blood of healthy female donors aged 29–31, who had undergone prior medical screening. Blood was drawn into EDTA-containing tubes and diluted 1:1 with PBS. The diluted blood was gently layered over Lymphoprep™ (StemCell Technologies, Vancouver, Canada) at a 2:1 ratio (blood:Lymphoprep™) and centrifuged at 2,500 rpm for 20 minutes at room temperature without braking. The plasma fraction from the top layer was carefully removed and placed on ice, while the intermediate mononuclear layer and Lymphoprep™ were discarded.

Neutrophils remaining in the pellet were freed from contaminating erythrocytes through treatment with 1× RBC lysis buffer (BioLegend, San Diego, CA, USA). Following a 10-minute incubation on ice with gentle agitation, samples were spun at 400 × g for 5 minutes and the supernatant containing lysed red blood cells was discarded. This lysis step was repeated once to ensure complete removal of erythrocytes. The resulting neutrophil population was resuspended in RPMI 1640 medium (Gibco, Waltham, MA, USA) supplemented with 2 mM L-glutamine and 10% autologous donor plasma. Cell viability was assessed using Trypan Blue exclusion.

Neutrophil infection with OMVs of P. gingivalis and S. aureus HG001

Neutrophils (5×10^5) were plated in each well of a 24-well plate using RPMI medium supplemented with 2 mM L-glutamine and 10% autologous serum. Some wells contained sterile coverslips to allow subsequent immunostaining, while others were left without coverslips for flow cytometry analysis. To examine the impact of P. gingivalis OMVs on neutrophil interactions with S. aureus, 5 µg of OMVs were pre-incubated with S. aureus for 15 minutes at 37°C before being added to the neutrophils. The dose of 5 µg OMVs per experiment was selected based on prior optimization experiments that tested a range of 1–10 µg. All samples were infected at a multiplicity of infection (MOI) of 15. Infections were carried out for 60 minutes at 37°C in 5% CO_2.

To remove extracellular bacteria, lysostaphin (25 µg/mL; AMBI Products, Rockville, MD, USA) was added, and the cultures were incubated for an additional 60 minutes under the same conditions [30]. Following incubation, cells on coverslips were fixed for immunostaining using 4% paraformaldehyde (PFA) for 15 minutes, followed by two washes with PBS. For flow cytometry, cells were harvested by gentle pipetting and transferred to 1.5 mL Eppendorf tubes. Cells were fixed with 4% PFA for 15 minutes at room temperature, washed once with PBS, and resuspended in 300 µL PBS. Samples were stored at 4°C until analysis by flow cytometry on the next day.

Antibodies against whole-cell P. gingivalis

Polyclonal antibodies targeting whole P. gingivalis W83 cells were generated by Eurogentec (Seraing, Belgium). For this purpose, the bacteria were first fixed with 1% PFA and thoroughly washed five times with PBS prior to immunization. A rabbit was selected for immunization based on pre-screening of its serum to ensure no cross-reactivity with P. gingivalis. The rabbit was then immunized following Eurogentec's established protocol. The resulting polyclonal antibodies were subsequently evaluated for specificity using both Western blotting and immunostaining.

Immunostaining

Coverslips containing fixed neutrophils and bacteria were first rinsed briefly with PBS. To allow antibody access to intracellular components, cells were permeabilized with 0.5% Tween-20 for 15 minutes at room temperature. Nonspecific binding sites were blocked by incubating the samples in 1% BSA for 1 hour at room temperature. Primary staining was performed with rabbit polyclonal antibodies directed against whole P. gingivalis for 1 hour at room temperature, followed by two washes with PBS.

Secondary detection employed goat anti-rabbit antibodies conjugated to AlexaFluor488, AlexaFluor647, or AlexaFluor555 (Invitrogen, Waltham, MA, USA) for 30 minutes in the dark at room temperature. During this step, neutrophil nuclei were counterstained with DAPI (Sigma-Aldrich), and in selected experiments, actin filaments were visualized using TRITC-phalloidin (Sigma-Aldrich). After two additional PBS washes, coverslips were mounted on Polylysine-coated slides (Thermo Fisher) using Mowiol 4-88 (Sigma-Aldrich). All handling steps were performed gently to maintain cell adhesion. Finally, the mounted samples were left to cure overnight at room temperature in the dark before imaging.

Confocal fluorescence microscopy

Image acquisition was performed on a Leica SP8 confocal microscope (Wetzlar, Germany, Leica Microsystems,), and the resulting data were processed and examined using LAS X software. For selected samples, two-dimensional confocal image stacks (Z-

stacks) were reconstructed into three-dimensional models using Imaris version 7.6.5 (Oxford Instruments, Abingdon, UK).

Flow cytometry

Quantification of neutrophils associated with S. aureus was carried out on a Cytoflex S flow cytometer (Beckman Coulter). GFP-expressing bacteria were excited with a 488-nm laser, and fluorescence was collected using a 525/40-nm emission filter. Flow cytometry datasets were evaluated in Kaluza Analysis Software (Beckman Coulter) following the gating procedure outlined previously [30]. From these analyses, the proportion of GFP-positive neutrophils and their mean fluorescence intensities were used to derive adherence and internalization indices according to established methods [31]. Data are expressed as the fold change in these indices when neutrophils were exposed to OMVs compared with untreated controls.

LDH cytotoxicity assay

The extent of neutrophil killing induced by S. aureus, either alone or in combination with outer membrane vesicles (OMVs) from Porphyromonas gingivalis, was evaluated by quantifying extracellular lactate dehydrogenase (LDH) release using the CyQUANT™ LDH Cytotoxicity Assay Kit (Thermo Fisher Scientific) according to the supplied protocol.

A total of 3.5×10^4 human neutrophils suspended in 100 µL of RPMI medium containing 2 mM L-glutamine and 10% autologous serum were dispensed into each well of a 96-well plate. The plate was then placed in a humidified incubator at 37°C with 5% CO_2 for 30 minutes to allow cells to adhere.

Wells assigned as maximum LDH release controls received 10 µL of 10× lysis buffer and were incubated for an additional 45 minutes under the same conditions. Spontaneous release controls were treated with 10 µL of sterile Milli-Q water.

Separately, S. aureus was incubated for 15 minutes at 37°C in supplemented RPMI medium either by itself or together with OMVs derived from P. gingivalis strains W83 or the PPAD-deficient mutant W83ΔPPAD. Neutrophils were subsequently challenged with these bacteria (or bacteria–OMV mixtures) at a multiplicity of infection (MOI) of 20, equivalent to 5.3×10^5 CFU and 0.35 µg of OMVs per well; the inoculum volume added was 10 µL. As a reference for strong bacterial leukotoxicity, Aggregatibacter actinomycetemcomitans strain 30R was included at MOI 100 [27].

Infection proceeded for 1 hour at 37°C in 5% CO_2. Afterward, 50 µL of cell-free supernatant from each well was transferred to a fresh 96-well plate, combined with 50 µL of the kit's reaction mixture, and incubated for 30 minutes at room temperature in the dark. The reaction was terminated by adding 50 µL of stop solution, and plates were gently tapped to mix.

Absorbance was measured at 490 nm (signal) and 680 nm (background). Background-subtracted values were used to calculate percent cytotoxicity with the standard formula:

$$\%\text{Cytotoxicity}=[(\text{treatedLDHactivity}-\text{SpontaneousLDHActivity})/(\text{MaximumLDHactivity}-\text{SpontaneousLDH activity})]\times 100 \quad (1)$$

S. aureus growth measurements

S. aureus cultures were grown in RPMI until they reached an OD_{600} of 0.5. The cultures were then diluted to an OD_{600} of 0.05, and 100-µl portions of this suspension were dispensed into a 96-well microtiter plate. Bacterial proliferation was monitored by recording OD_{600} values every 15 minutes at 37°C with continuous shaking using a Synergy Two multi-mode plate reader (BioTek Instruments, Winooski, VT). When the cultures entered either the early or late exponential phase, 5 µg of OMVs derived from P. gingivalis W83 or its PPAD-deficient mutant (W83ΔPPAD) were introduced into the wells. Growth trajectories were plotted using GraphPad Prism 8.0 (GraphPad Software, San Diego, CA), and the influence of OMV addition on actively dividing bacteria was evaluated by comparing OMV-treated samples with untreated controls. Each condition was tested in triplicate, and mean values were used to generate the curves.

To determine whether gingipain activity contributed to OMV-dependent staphylococcal aggregation, gingipain inhibitors (final concentration: 100 µM) were added simultaneously with the OMVs. The inhibitors employed were Cathepsin B inhibitor II for Kgp (Merck, Kenilworth, NJ) and Leupeptin for Rgp (Sigma-Aldrich). To test whether S. aureus could adapt to OMV-induced aggregation, cells that had interacted with OMVs and reached stationary phase were collected and subjected to a subsequent growth cycle in the presence or absence of P. gingivalis OMVs.

Aggregate formation by GFP-labeled S. aureus was visualized using an Amersham Typhoon NIR imager, which allowed detection of wells containing aggregated or dispersed bacteria. To determine the spatial distribution of OMVs within the bacterial clusters, aggregated material was transferred to a fresh 96-well plate, immunostained for OMVs, and examined by confocal fluorescence microscopy as described previously.

Medical ethical approval

Healthy volunteers donated blood for this study under a protocol approved by the Medical Ethical Committee of the University Medical Center Groningen (Metc2012-375). Written consent was obtained from all participants prior to donation, and all procedures followed the ethical principles of the Helsinki Declaration.

Statistical analyses

All datasets were processed and visualized in GraphPad Prism 8.0 (GraphPad Software). Statistical comparisons were carried out using a two-tailed paired Student's t-test, and differences were regarded as statistically meaningful when the P-value was ≤ 0.05.

Results and Discussion

Reduced CFU counts upon incubation of S. aureus with OMVs of P. gingivalis

Given the established association between SAB and rheumatoid arthritis [9, 10] and the proposed involvement of P. gingivalis in RA development [11], we examined how OMVs produced by P. gingivalis influence S. aureus behavior, with particular attention to PPAD and the gingipains—virulence factors linked to RA-related processes. For this purpose, S. aureus HG001 was exposed for 90 minutes to OMVs derived either from the P. gingivalis W83 wild-type strain or from its PPAD-deficient variant (W83ΔPPAD), while control samples received only PBS. RPMI medium was used throughout these experiments, as prior transcriptomic analyses demonstrated that S. aureus HG001 exhibits a gene expression pattern in RPMI that closely resembles its transcriptional profile during growth in human plasma [32].

Following incubation, bacteria were plated on blood agar. A marked reduction in colony-forming units was observed when S. aureus had been incubated with OMVs compared to the PBS-treated control **(Figure 1)**. To assess whether gingipain activity contributed to this reduction, we included leupeptin and cathepsin B inhibitor II—compounds that inhibit RgpA/B and Kgp, respectively [33]. These inhibitors did not prevent the OMV-induced drop in CFU counts **(Figure 1)**. However, when S. aureus was exposed to OMVs from the W83ΔPPAD mutant in the presence of the gingipain inhibitors, the decline in CFUs appeared somewhat less pronounced **(Figure 1)**.

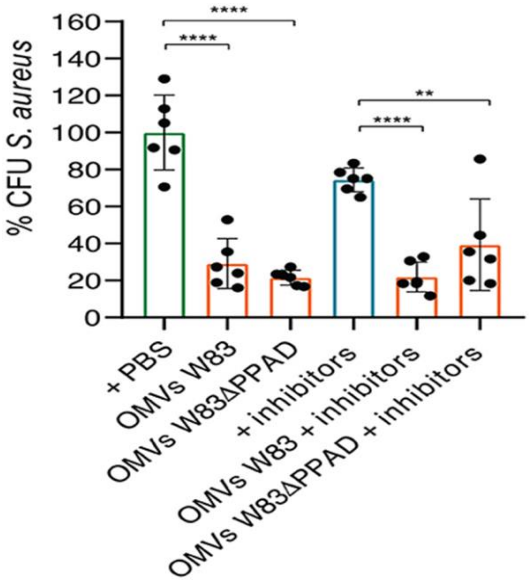


Figure 1. P. gingivalis OMVs diminish recoverable S. aureus CFUs.

For this assay, S. aureus HG001 was exposed for 90 minutes to OMVs derived from P. gingivalis W83 or the PPAD-deficient mutant W83ΔPPAD. Gingipain inhibitors—Cathepsin B inhibitor II (blocking Kgp) and Leupeptin (blocking RgpA/B)—were included in parallel samples to assess their influence. All conditions were evaluated relative to S. aureus treated only with PBS. Values shown represent means with standard deviations from three independent biological experiments, each performed in duplicate. Statistical differences were assessed using two-tailed, unpaired Student's t-tests. **, $P < 0.01$; ****, $P < 0.0001$.

Outer membrane vesicles (OMVs) from P. gingivalis trigger S. aureus to cluster together, and this effect relies on both PPAD and gingipain activity.

To determine whether the reduced CFU counts of S. aureus were the result of OMV-mediated killing or instead reflected bacterial clumping, as previously described by Kamaguchi and colleagues [17], we monitored S. aureus growth in the presence of P. gingivalis OMVs. During exponential growth, S. aureus HG001 was exposed to OMVs isolated from P. gingivalis W83 or its PPAD-knockout derivative, W83ΔPPAD. As illustrated in **Figure 2a**, introducing OMVs caused a short-lived decline in OD600 values. However, the cultures subsequently recovered and eventually reached optical densities similar to untreated controls, indicating that OMVs did not exert a bactericidal effect. Instead, the OD600 traces displayed pronounced oscillations after OMV addition, suggesting the formation of bacterial aggregates.

To confirm aggregation, we imaged the wells for GFP fluorescence emitted by the HG001 strain carrying

plasmid pJL-sar-GFP. Aggregated fluorescent bacterial clusters were readily observed in OMV-treated samples **(Figure 2b)**. Immunostaining of material collected from these wells further demonstrated that the OMVs were embedded within the S. aureus aggregates **(Figure 2b)**. In contrast, supplementing cultures of P. gingivalis W83 or W83ΔPPAD with their own OMVs did not noticeably influence the growth behavior of either strain **(Figures 2c and 2d)**.

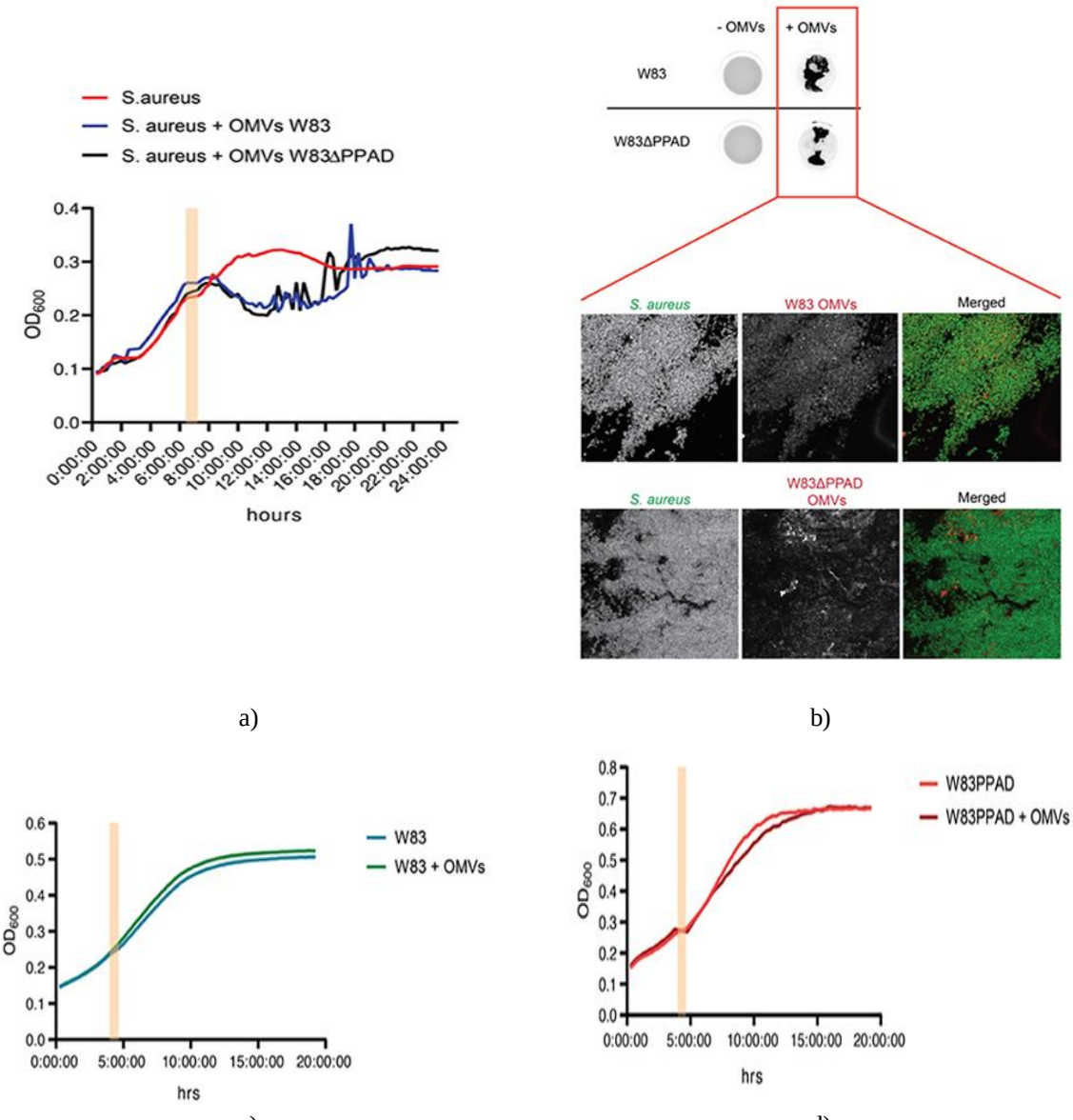
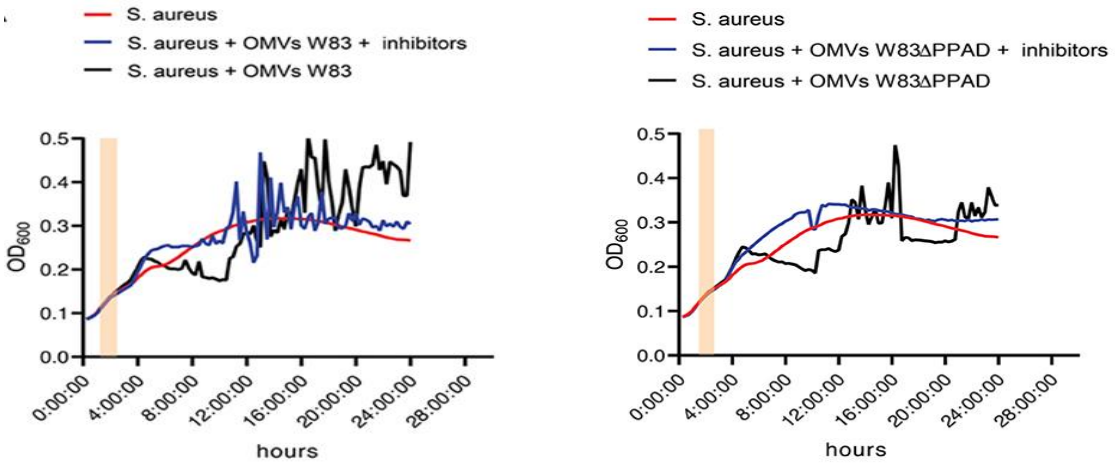


Figure 2. Outer membrane vesicles (OMVs) from P. gingivalis promote clumping of S. aureus in a PPAD-dependent fashion. (a) Real-time growth monitoring of S. aureus HG001 in 96-well plates. Mid-log phase cultures were supplemented (or not) with OMVs isolated from wild-type P. gingivalis W83 or the isogenic W83ΔPPAD mutant. Sharp drops and irregular oscillations in OD600 signal bacterial clumping. (b) Macroscopic appearance of culture wells after OMV challenge and confirmatory confocal microscopy. S. aureus constitutively expressing GFP (pJL-sar-GFP plasmid) forms large clusters that precisely overlap with antibody-labeled OMVs (scale bars represent 50 μm). (c, d) Growth profiles of P. gingivalis W83 and W83ΔPPAD strains with or without addition of their own OMVs (time of addition highlighted in pink). Experiments included four independent biological replicates, each with one technical replicate.

OMVs produced by the PPAD-knockout strain triggered noticeably weaker clumping than those from the parental strain **(Figure 2b)**, indicating that PPAD actively contributes to the phenomenon.

Because gingipains are essential for the maturation and activity of PPAD and play broad roles in P. gingivalis pathogenesis, we next assessed whether proteolytic activity of gingipains is also required **(Figure 3)**. Treatment with the gingipain inhibitors leupeptin and cathepsin B inhibitor II markedly reduced clumping, resulting in much smoother growth curves **(Figures 3a and 3b)** and visibly fewer aggregates in the wells **(Figure 3c)**. The effect was particularly striking with PPAD-deficient OMVs, where inhibitor treatment nearly eliminated aggregation altogether **(Figures 3b and 3c)**.

These findings establish that full aggregation of S. aureus by P. gingivalis OMVs requires coordinated action of both PPAD and gingipains. The partial phenotypes observed with either PPAD deletion or gingipain blockade alone reveal a clear synergistic relationship, entirely consistent with previous reports that gingipains must first process substrates to allow subsequent citrullination by PPAD [11, 14].



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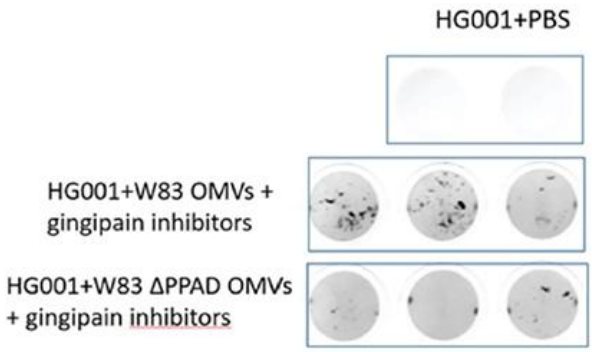


c)

Figure 3. Inhibition of gingipains or deletion of PPAD dramatically reduces OMV-induced clumping of S. aureus. (a) Real-time growth monitoring of S. aureus HG001 supplemented with OMVs from wild-type P. gingivalis W83, either untreated or pretreated with the gingipain inhibitors cathepsin B inhibitor II and leupeptin. (b) Parallel experiment using OMVs harvested from the P. gingivalis W83ΔPPAD mutant, with or without the same gingipain inhibitors. (c) Photographs of the 96-well plates at the end of the experiment, showing the extent of visible bacterial aggregates under each condition. The time of OMV addition is indicated by pink shading on the growth curves. All conditions were tested in four independent biological replicates with one technical replicate each.

The clumping of S. aureus triggered by P. gingivalis OMVs proved completely reversible.

To determine whether OMV-induced aggregation of S. aureus is reversible, bacteria that had previously formed aggregates in the presence of P. gingivalis OMVs were collected and re-cultured either in the absence or presence of OMVs. As illustrated in **Figure 4**, the previously aggregated S. aureus grew normally when OMVs were omitted, while re-exposure to OMVs from either P. gingivalis W83 or W83ΔPPAD triggered aggregation once again. These results

indicate that the aggregation induced by P. gingivalis OMVs is a fully reversible phenotype.

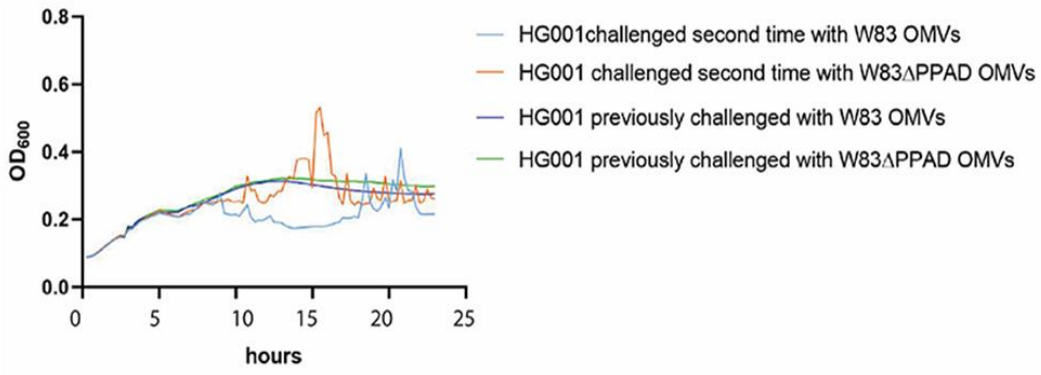


Figure 4. The clumping phenotype induced by P. gingivalis OMVs is entirely reversible

S. aureus HG001 was initially incubated with OMVs from wild-type P. gingivalis W83 or the isogenic W83ΔPPAD strain until strong aggregation developed. At the 5-hour mark, the clumped bacteria were pelleted by low-speed centrifugation were gently resuspended and transferred into fresh RPMI medium. Subcultures were then split: one set received no additional OMVs, while the other received a fresh dose of the same OMVs.

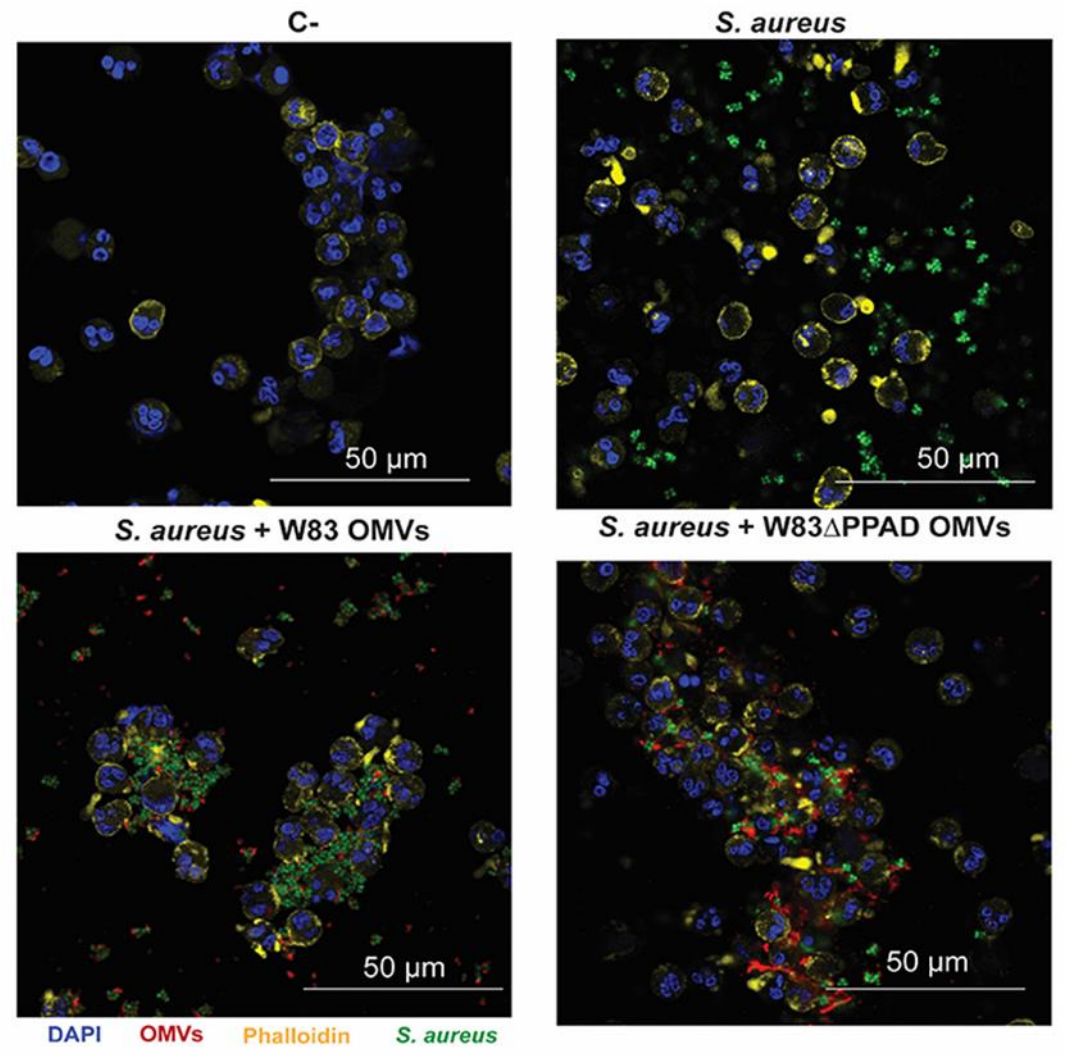
Cultures designated "previously exposed" were grown without further addition of OMVs. These rapidly returned to normal, non-aggregating growth patterns indistinguishable from never-exposed controls, confirming full reversibility of the phenotype upon OMV removal. All experiments included four independent biological replicates with one technical replicate per condition.

P. gingivalis OMV-induced neutrophil adhesion and internalization of S. aureus

Building on the known connection between rheumatoid arthritis and SAB, we investigated whether OMVs from P. gingivalis influence how S. aureus interacts with human neutrophils, which serve as a primary innate defense against both pathogens [1, 31]. For this purpose, S. aureus HG001 was first incubated with OMVs from either P. gingivalis W83 or the PPAD-deficient mutant W83ΔPPAD, then introduced to primary human neutrophils. Confocal microscopy was used to examine these interactions. In **Figure 5**, neutrophil nuclei are shown in blue (DAPI), actin filaments in yellow (phalloidin-TRITC), S. aureus in green (GFP), and OMVs in red (antibody-labeled). OMV exposure from both W83 and W83ΔPPAD clearly induced aggregation of S. aureus and promoted bacterial uptake by neutrophils **(Figure 5a)**.

Flow cytometric analyses confirmed that OMV-treated S. aureus displayed enhanced binding to neutrophils and increased internalization compared with untreated bacteria **(Figure 6a)**. Notably, although OMVs coated the S. aureus surface prior to phagocytosis, the vesicles themselves were not internalized alongside the bacteria **(Figures 5b and 5c)**. To assess whether OMVs affected neutrophil viability, an LDH release assay was performed. No cytotoxicity was observed when neutrophils were exposed to S. aureus, regardless of OMV treatment. In contrast, exposure to the leukotoxic oral bacterium A. actinomycetemcomitans 30R caused substantial neutrophil death and LDH release [27] **(Figure 6b)**.

Together, these results indicate that P. gingivalis OMVs enhance neutrophil-mediated phagocytosis of S. aureus. This effect could serve to remove microbial competitors or act as an immune distraction, diverting neutrophils away from targeting P. gingivalis.



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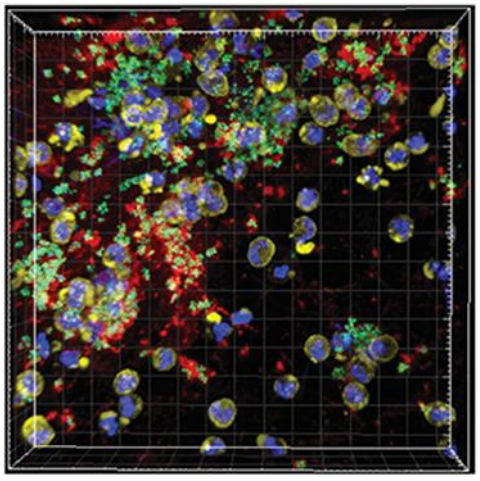

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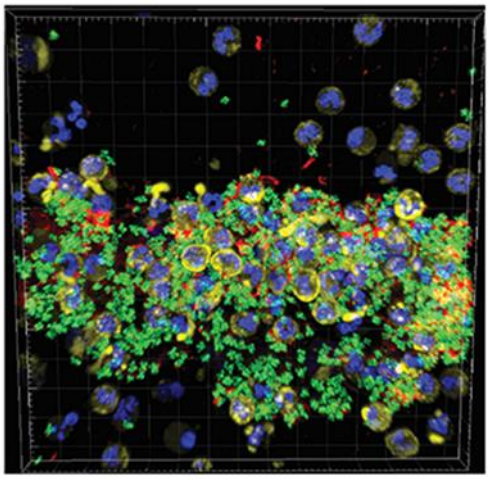
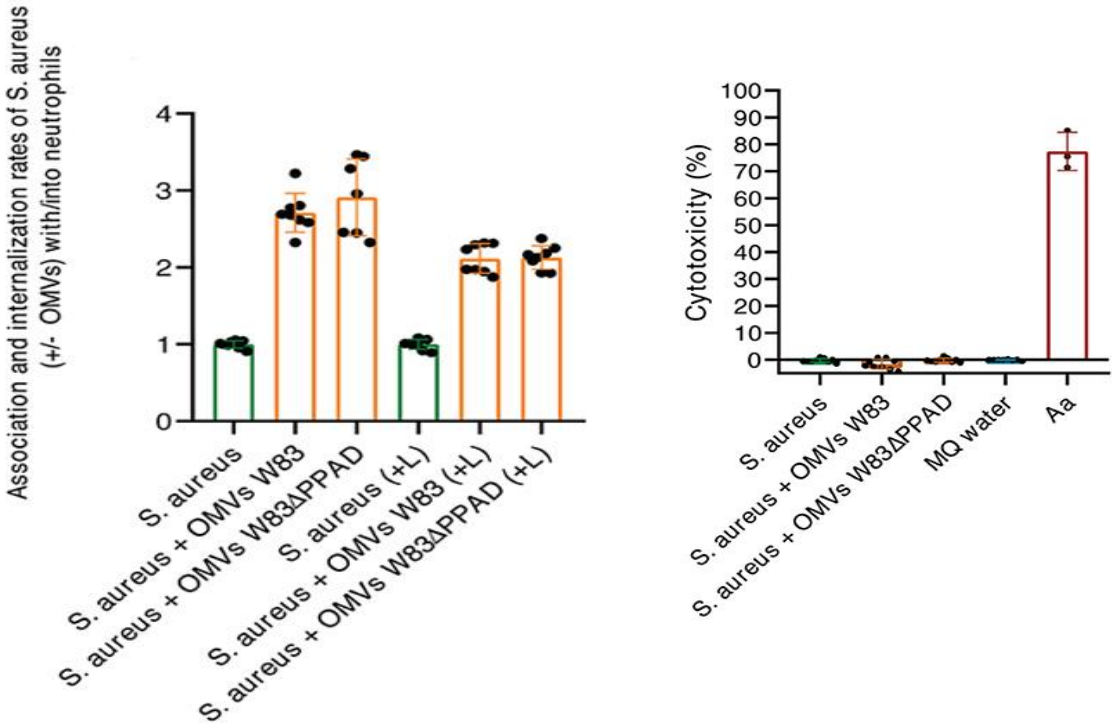


c)

Figure 5. Neutrophil uptake and close contact with S. aureus after treatment with P. gingivalis outer membrane vesicles (OMVs). (a) Example confocal images of human neutrophils infected with S. aureus strain HG001 that had been pre-incubated with P. gingivalis OMVs. (b, c) 3D-rendered views from confocal z-stacks showing neutrophils exposed to S. aureus together with OMVs isolated from wild-type P. gingivalis W83 (b) or from the PPAD-deletion mutant W83ΔPPAD **(C).** DNA in neutrophil nuclei was labeled with DAPI (blue), F-actin was stained with TRITC-conjugated phalloidin (red), S. aureus cells expressed GFP was

constitutively expressed from plasmid pJL-sar-GFP (green), and OMVs were detected by immunofluorescence using specific antibodies (scale bars = 50 μm). Experiments were independently repeated at least two times.



a) b)

Figure 6. Effects of P. gingivalis OMVs on S. aureus phagocytosis and neutrophil survival. Fold changes in the association and internalization of S. aureus in the presence of OMVs compared with untreated bacteria. L, lysostaphin. (b) Neutrophil cytotoxicity measured via LDH release after exposure to S. aureus treated with OMVs from either P. gingivalis W83 or W83ΔPPAD. Aggregatibacter actinomycetemcomitans 30R (a) served as a positive control for neutrophil death, and MilliQ water was used to account for spontaneous LDH release. Data are presented as mean ± SD from three independent biological replicates, each with two technical replicates.

Conclusion

Staphylococcus aureus is frequently detected in subgingival biofilms and saliva, prompting earlier investigations into how P. gingivalis strain ATCC 33277 and its outer membrane vesicles affect this species [17-21]. Using the highly virulent P. gingivalis strain W83, we now demonstrate two previously unrecognized phenomena: OMV-driven aggregation of S. aureus is completely reversible upon removal of the vesicles rapidly restores normal planktonic growth. Aggregation depends on the combined activity of gingipains and PPAD.

The participation of PPAD is particularly noteworthy, because prior work had implicated only gingipains, attributing their effect to hemagglutinin/adhesin domains [17, 34]. Our data reveal a clear synergistic requirement: gingipains first cleave proteins to generate C-terminal arginine residues, which are then citrullinated by PPAD [11, 14]. This strongly implies that protein citrullination on the surface of OMVs, S. aureus, or both is a key mechanistic driver of aggregation. Identifying the exact molecular partners on each side will be an important next step.

A second major finding is that OMV-induced clumping markedly enhances uptake of S. aureus by human neutrophils. From the perspective of P. gingivalis, this may represent a clever strategy to eliminate a competing flora within the dysbiotic pocket. For the human host, however, the consequences could be serious: S. aureus is notorious for surviving inside neutrophils and exploiting phagocytes as vehicles for systemic dissemination [1]. Neutrophil-mediated transport into the bloodstream could therefore provide a biologically plausible route by which severe periodontal infection contributes to life-threatening staphylococcal bacteremia, offering a potential mechanistic link between rheumatoid arthritis (where P. gingivalis-driven autoimmunity via PPAD is well

documented) and the elevated risk of S. aureus bloodstream infection observed in RA patients [9, 10]. These connections remain hypothetical at present. Rigorous follow-up studies are needed to examine survival of aggregated S. aureus after OMV-facilitated phagocytosis, as well as the broader tripartite interplay among P. gingivalis, S. aureus, and host immunity. Such work may ultimately uncover new preventive or therapeutic strategies that simultaneously target periodontal disease and invasive staphylococcal infection.

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