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Acceleration of the formation of biofilms on contact lens surfaces in the presence of neutrophil-derived cellular debris is conserved across multiple genera

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Purpose: We have previously shown that invasive strains of *Pseudomonas aeruginosa* exploit the robust neutrophil response to form biofilms on contact lens surfaces and invade the corneal epithelium. The present study investigated the ability of multiple bacterial genera, all commonly recovered during contact lens–related infectious events, to adhere to and form biofilms on contact lens surfaces in the presence of neutrophils.
Methods: Five reference strains from the American Type Culture Collection were used: *P. aeruginosa, Serratia marcescens, Stenotrophomonas maltophilia, Staphylococcus aureus,* and *Staphylococcus epidermidis*. Each bacterial strain was incubated overnight with or without stimulated human neutrophils in the presence of an unworn contact lens. Standard colony counts and laser scanning confocal microscopy of BacLight-stained contact lenses were used to assess bacterial viability. Three-dimensional modeling of lens-associated biofilms with Imaris software was used to determine the biofilm volume. Lenses were further examined using scanning electron microscopy.
Results: Less than 1% of the starting inoculum adhered to the contact lens surface incubated with bacteria alone. There were no differences in adhesion rates to contact lens surfaces between bacteria in the absence of neutrophils for either the Gram-negative or Gram-positive test strains. Bacterial adhesion to contact lens surfaces was accelerated in the presence of human neutrophils for all test strains. This effect was least evident with *S. epidermidis.* There was also an increase in the number of viable bacteria recovered from contact lens surfaces ($p<0.001$ for the Gram-negative and Gram-positive test strains, respectively) and in biofilm volume ($p<0.001$ for the Gram-negative test strains, $p = 0.005$ for *S. aureus*).
Conclusions: These results show that in addition to *P. aeruginosa*, other bacteria commonly encountered during contact lens wear possess the capacity to utilize neutrophil-derived cellular debris to facilitate colonization of the lens surface. These data suggest that this phenomenon is conserved among multiple genera. Thus, during contact lens wear, the presence of inflammation and the accumulation of neutrophil debris under the posterior lens surface likely contribute to colonization of the lens. Further studies are needed to correlate these findings with risk for infection in an animal model.

Contact lenses are one of the most commonly used medical devices, with an estimated 150 million contact lens users worldwide [1]. Contact lens–related microbial keratitis is the most severe and visually devastating complication associated with contact lens wear, with an annualized incidence of 4 per 10,000 wearers per year for daily wear and rising to 20 per 10,000 wearers per year for those electing to use extended wear [2-6]. Various strategies have been implemented to promote safer contact lens wear. These strategies include the introduction of disposable contact lenses in the early 1990s and the development of higher oxygen-permeable silicone hydrogel contact lens materials. Despite more than three decades of research and significant shifts in lens materials and wearing modalities, the annualized incidence of contact lens–related microbial keratitis remains unchanged, and the overall incidence of non-infectious inflammatory events has doubled [6,7].

Previous epidemiological studies identified *Pseudomonas aeruginosa* as the primary causative agent in contact lens–related corneal infection [2-5,8-10]. *Staphylococcus aureus,* coagulase-negative *Staphylococci,* and *Serratia marcescens* ave also been identified as common causative agents in contact lens–related corneal infection [11]. *Stenotrophomonas maltophilia* is an emerging pathogen in contact lens–related microbial keratitis and is commonly found in contact lens storage cases [12]. Our laboratory has shown that invasive corneal isolates of *P. aeruginosa,* when trapped under the lens surface during wear, exploit the robust subclinical inflammatory response to enhance colonization on the lens surface and facilitate lipid raft-mediated uptake into the corneal epithelium [13]. This uptake occurs as a result of charged interactions between extracellular DNA and F-actin released by dying neutrophils, forming cellular scaffolds that facilitate adherence and colonization [13-17]. Although other studies have shown that neutrophil interactions with bacteria

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are not always associated with cell death, our previous work using live/dead staining showed that viable neutrophils are not present on contact lens surfaces when they are incubated overnight with *P. aeruginosa* [13,18]. Further, the use of necrotic neutrophil debris from repeated freeze–thaw cycles has been shown to promote the same biofilm-forming effects as stimulated neutrophils [16].

The formation of a biofilm has been reported on contact lenses recovered from humans with active microbial keratitis [19]. In addition, laboratory studies in a rat contact lens model have shown that *P. aeruginosa* biofilms form on the posterior surface of the contact lens during an infectious event [20]. Our laboratory has further shown disruption of *P. aeruginosa* neutrophil-enhanced biofilms during contact lens wear inhibits bacterial uptake in to the rabbit corneal epithelium, demonstrating a clear reduction in the infectious bioburden in the lens-wearing eye [13]. Similar to *P. aeruginosa*, all four additional test pathogens have the capacity to form biofilms; however, the response of these test pathogens to neutrophil-enhanced colonization is unknown [21-23].

The goal of this study is to extend our previous observations using *P. aeruginosa* and investigate the ability of five species of bacteria, all commonly recovered during a contact lens–related adverse event, to utilize neutrophil-derived cellular debris scaffolds to colonize lens surfaces. To test this phenomenon, we used a standard, commercially available silicone hydrogel contact lens. The ability of neutrophils (short-lived inflammatory cells) to facilitate colonization may play an important role in the development of contact lens–related corneal infection and other lens-related adverse events during soft contact lens wear.

METHODS

Bacterial strains: Five reference strains from the American Type Culture Collection (ATCC, Manassas, VA) were selected for use in this study: *Pseudomonas aeruginosa* (ATCC 9027), *Staphylococcus epidermidis* (ATCC 35984), *Staphylococcus aureus* (ATCC 6538), *Stenotrophomonas maltophilia* (ATCC 13637), and *Serratia marcescens* (ATCC 13,880). Bacteria were maintained in a 50% v:v glycerol stock at −80 °C. For the experiments, the bacteria were grown on tryptic soy agar (TSA; Sigma-Aldrich, St. Louis, MO) plates overnight at 37 °C. A single clone was selected and grown overnight on a TSA slant at 37 °C. The bacteria were then suspended in PBS to a concentration of approximately 10^8 colony forming unit (CFU)/ml using a spectrophotometer (SmartSpec Plus; Bio-Rad, Hercules, CA). Absorbance was 0.3 at 650 nm, and the resultant bacterial suspension was diluted to approximately 10^6. All inocula were confirmed with serial plating for determination of the CFUs.

Neutrophil isolation: Whole blood was collected from healthy human volunteers by venipuncture as we have previously reported [13]. All procedures were approved by the Institutional Review Board at UT Southwestern Medical Center, performed according to the tenets of the Declaration of Helsinki and adhered to the ARVO statement on human subjects. Each subject signed an informed consent before participating in this study. Before each experiment, blood was collected using 4.5 ml vacutainers containing 3.2% citrate (BD, Franklin Lakes, NJ). Three vials of blood were combined into a single 50 ml conical tube, and neutrophils were then isolated using a Percoll gradient to separate the plasma [24]. To prevent non-specific activation of neutrophils, all isolation procedures were performed at room temperature. Pooled samples underwent centrifugation for 20 min at 300 ×*g*, and the top layer of platelet-rich plasma was removed, followed by an additional centrifugation step at 2,500 ×*g* for 15 min to collect the platelet-poor plasma (PPP). Using the remaining sample from the initial centrifugation step, 5 ml of 6% dextran and 0.9% saline were added to bring the total sample volume to 50 ml and then mixed with gentle inversion. After 30 min, the leukocyte-rich layer was removed and centrifuged for 6 min at 275 ×*g*. The pellet was then resuspended in the PPP. Solutions containing 42% and 51% Percoll (Sigma-Aldrich, St. Louis, MO) in the PPP were added and centrifuged at 275 ×*g* for 10 min. Neutrophils, which are present at the interface between the two Percoll layers, were carefully collected and washed using the PPP followed by centrifugation at 275 ×*g*. The neutrophils were then resuspended in Roswell Park Memorial Institute Media (RPMI, Sigma-Aldrich) containing 2% heat-inactivated PPP (HIPPP), and the final concentration of cells was determined using a hemacytometer. Two percent HIPPP was generated by incubation of the PPP in a water bath at 56 °C for 30 min followed by centrifugation. The neutrophils were then stimulated for 1 min using 60 ng/ml (25 nM) of phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich), a neutrophil-activating agonist. Activated neutrophils were then pelleted with centrifugation and resuspended in RPMI and 2% HIPPP to reach a final neutrophil concentration of 16.6×10^6 cells/ml. The neutrophils were then incubated at 37 °C for 2 h before the bacteria were added.

Contact lens incubation: Unworn lotrafilcon B (Alcon Laboratories, Ft. Worth, TX) soft contact lenses with a base curve of 8.6, a diameter of 14.2, and a power of −0.50 were used in this study. In a sterile hood, each lens was cut into four equal parts using sterile tweezers and a sterile razor blade and individually placed into single wells in a 24-well plate. Lenses

were cut into four equal portions to allow for flattening of the lens for optimal imaging after the incubation period. Contact lenses were incubated with bacteria in the presence of neutrophils in RPMI at a multiplicity of infection (MOI) of 1. Lenses incubated with bacteria alone in RPMI were used as non-neutrophil-treated controls. Lenses incubated in neutrophils alone were also included as additional controls to ensure no cross-contamination occurred. The contact lenses were then incubated overnight for approximately 18 h at 37 °C. A total of three contact lens sections were used for each bacteria species or bacteria species and neutrophil combination. All experiments were performed in triplicate.

Laser scanning confocal microscopy: After overnight incubation at 37 °C, the contact lenses were stained using a LIVE/DEAD BacLight Bacterial Viability Kit (Life Technologies, Grand Island, NY). Each lens was stained with a 3 µl mixture containing 1.5 µl of SYTO 9 and 1.5 µl propidium iodide (PI) for 15 min at room temperature. The contact lenses were washed and mounted on separate 35 mm diameter glass-bottom MatTek culture dishes (MatTek Corp, Ashland, MA) using a 50:50 v:v PBS/glycerol mixture and coverslipped. This prevented dehydration of the contact lenses during scanning. The contact lenses were viewed using a Leica SP2 laser scanning confocal microscope (Leica Microsystems, Heidelberg, Germany). To minimize any spectral overlap between the emission channels, the images were sequentially scanned. Image stacks were acquired from three representative areas of the surface of each contact lens. The image stacks were reconstructed three dimensionally using Imaris software (Bitplane, South Windsor, CT). For analysis of the biofilm volume, isosurfaces were applied to each independent channel using the surface function in Imaris. Isosurfaces were manually adjusted to optimize the 3D representation of the biofilm. The cubic volume of the total biofilm was automatically calculated by Imaris from the generated isosurfaces [25].

Scanning electron microscopy: In a subsequent experiment, following overnight incubation at 37 °C, the soft contact lenses were fixed in 2.5% glutaraldehyde/0.1 M cacodylate buffer pH 7.4. The lenses were processed in the Electron Microscopy Core at the University of Texas Southwestern Medical Center according to the following protocol. Briefly, the lenses were washed in 0.1 M cacodylate pH 7.4 and then subjected to secondary fixation using 1% osmium. After rinsing with water, the lenses underwent an ethanol dehydration series followed by drying with hexamethyldisilazane (Sigma-Aldrich). Lenses were air dried, mounted on aluminum stubs, and sputter-coated with gold-palladium in a Cressington 108 Auto Sputter Coater (Cressington Scientific Instruments, Watford, UK). Lenses were then imaged with a Zeiss Sigma VP field emission scanning electron microscope (Zeiss, Jena, Germany).

Viable bacterial quantification: After overnight incubation, each contact lens was removed from the 24-well plate and washed in a sterile well plate containing PBS with gentle agitation to dislodge any loosely adherent bacteria. Next, the bacteria were removed from each lens by placing the lens in sterile PBS in a 1.5 ml Eppendorf tube. The tube was then placed in water and subject to water sonication at 50–60 Hz for 1 min followed by vortexing on high for 2 min to eliminate any residual clumping. Preliminary testing in our laboratory has shown that this method optimizes the yield of viable bacteria from the lens surface. The solution was serially diluted with PBS and plated on TSA plates in triplicate for each dilution. The plates were then incubated at 37 °C overnight, and colony counts were obtained for each bacterial test strain with and without neutrophils. Each experiment was performed in triplicate.

Statistical analysis: Statistical analysis was performed using Sigma Plot 11.0 (Systat Software, Inc., San Jose, CA). All data are expressed as mean ± standard deviation. Normality and variance were tested using the Shapiro–Wilk normality test and the Kolmogorov–Smirnov equal variance test. To assess differences between Gram-positive bacteria, a Student *t* test was used. To assess differences between the inocula and adherent bacteria for Gram-negative and Gram-positive strains, a one-way ANOVA and a Student–Newman–Keuls post hoc comparison test were used. To compare differences between Gram-negative strains with and without neutrophils and Gram-positive strains with and without neutrophils, a two-way ANOVA was used followed by a Student–Newman–Keuls post hoc test for multiple comparisons. A p value of less than 0.05 was considered statistically significant.

RESULTS

Bacterial adhesion without neutrophils: After dilution to 10^6 followed by an overnight (18 h) incubation period, less than 1% of the initial inoculum adhered to the contact lens surfaces. There were no differences in bacterial adherence to the lens surface in the absence of neutrophils between the two Gram-positive test strains ($p = 0.574$, Student *t* test, Figure 1A) or among the three Gram-negative strains ($p = 0.091$, one-way ANOVA, Figure 1B).

Gram-positive bacterial adhesion in the presence of neutrophils: Scanning electron microscopy was used to visualize the ultrastructure of adherent bacteria to the contact lens surface with and without stimulated neutrophils. For *S. aureus*, in the absence of neutrophils, small, round clusters were visible

(Figure 2A). Coincubation of *S. aureus* with neutrophils resulted in much greater lens colonization along with obvious extracellular matrix production (Figure 2B). Similar to *S. aureus*, *S. epidermidis* produced small round clusters on the lens surface; however, this was not greatly altered in the presence of neutrophils (Figure 2C,D). Laser scanning confocal microscopy imaging of the stained bacteria showed similar findings (Figure 2E–H). A large percentage of the adherent bacteria was viable with BacLight staining. Colony counts of adherent bacteria revealed a 3-log increase in viable bacteria recovered from the lens surface for *S. aureus* (2.5×10^5 +/− 2.5×10^4 CFU/ml for bacteria alone compared with 1.6×10^8 +/− 7.1×10^6 CFU/ml with neutrophils, Figure 2I). This result was statistically significant ($p<0.001$; two-way ANOVA). There was a similar, but smaller, increase for *S. epidermidis* (3.6×10^5 +/− 1.8×10^5 CFU/ml compared with 1.9×10^7 +/− 2.1×10^6 CFU/ml, $p = 0.008$). The neutrophil-mediated increase in *S. aureus* adherence was statistically significantly greater than for *S. epidermidis* ($p<0.001$).

Gram-negative bacterial adhesion in the presence of neutrophils: Scanning electron microscopy of the lenses incubated with each individual Gram-negative bacterium in the absence of neutrophils showed sparse bacteria localized in small focal clumps across the lens surface (Figure 3A,C,I). When the

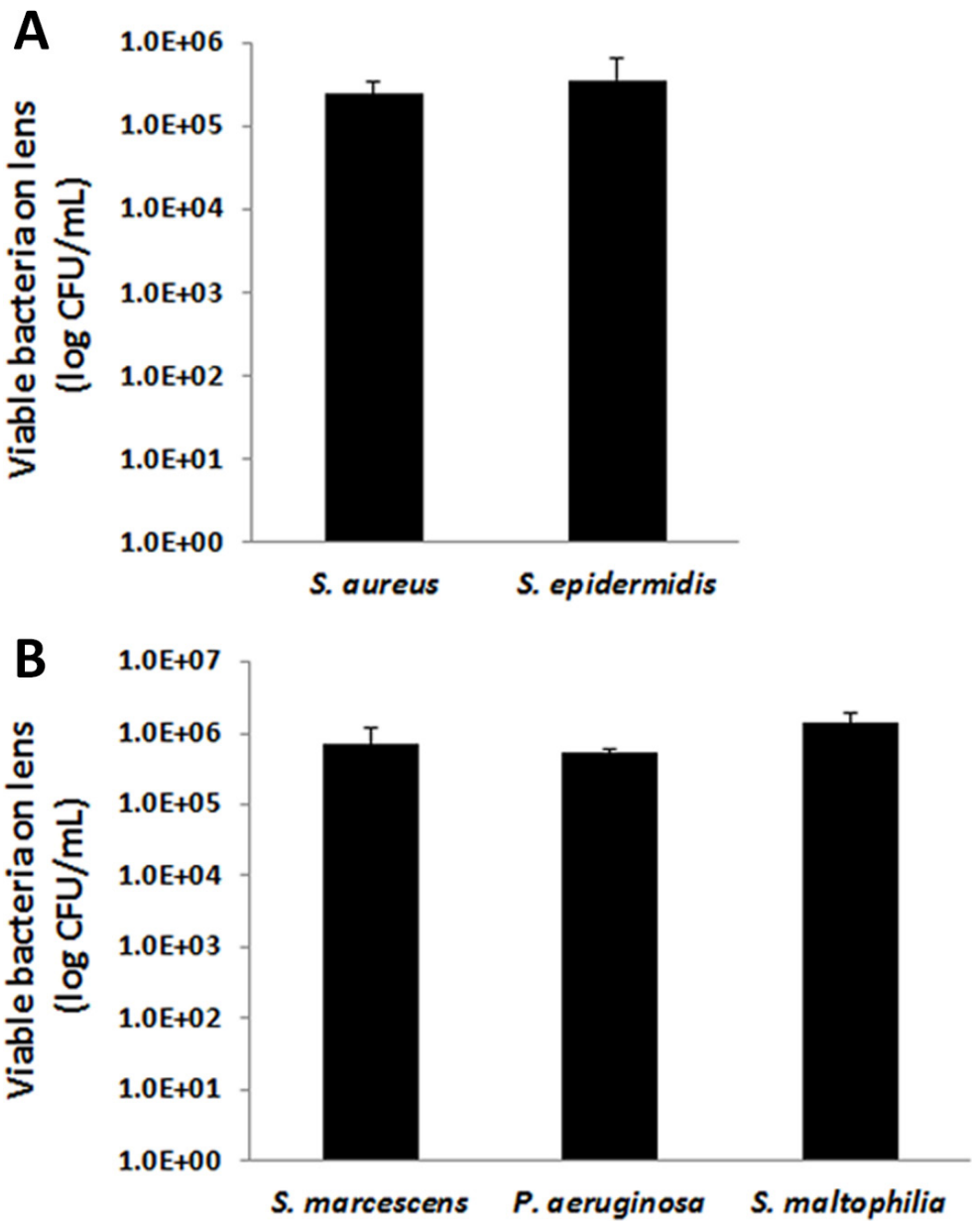

Figure 1. Bacterial adhesion in the absence of neutrophils. **A**: Without neutrophils, there was no statistically significant difference in the viable inoculum adherent to the lens surface after 18 h of incubation between Gram-positive test strains ($p = 0.574$, Student *t* test, $n = 3$). **B**: There were no statistically significant differences in the bacteria recovered from the lens surface after 18 h of incubation between test strains ($p = 0.091$, one-way ANOVA, $n = 3$).

lenses were incubated with neutrophils, the density and the architecture of the colonized bacteria were dramatically enhanced (Figure 3B,D,J). For *P. aeruginosa*, a large amount of extracellular matrix was visible across the surface of the clumped bacteria (Figure 3D). These findings were consistent with the 3D confocal microscopy data showing an increase in bacterial colonization on the lens surfaces for all three strains (Figure 3E–L). The majority of the bacteria on the lenses incubated in neutrophils were viable when viewed using BacLight staining. Colony counts of the viable bacteria recovered from the lens surface increased by several log units for all three Gram-negative test strains (Figure 3M). The CFU counts for *S. marcescens* increased by 3 log units from 6.9×10^5 +/− 3.05 $\times 10^5$ CFU/ml to 2.1×10^8 +/− 4.0×10^7 CFU/ml ($p<0.001$, two-way ANOVA). The CFU counts for *P. aeruginosa* also increased 3 log units from 5.4×10^5 +/− 2.4×10^4 CFU/ml to 3.5×10^8 +/− 2.4×10^7 CFU/ml ($p<0.001$). The CFU counts for *S. maltophilia* increased but by only 2 log units (1.4×10^6 +/− 2.9×10^5 to 1.6×10^8 +/− 1.7×10^7 CFU/ml, $p<0.001$). The overall increase in lens colonization by *P. aeruginosa* in the presence of neutrophils was statistically significantly greater than that for *S. marcescens* or *S. maltophilia* ($p<0.001$).

3D analysis of biofilm volume for Gram-positive and Gram-negative bacteria: Using the surface function in Imaris, the 3D volume of the lens-colonized bacteria in the absence and presence of neutrophils was determined [25].

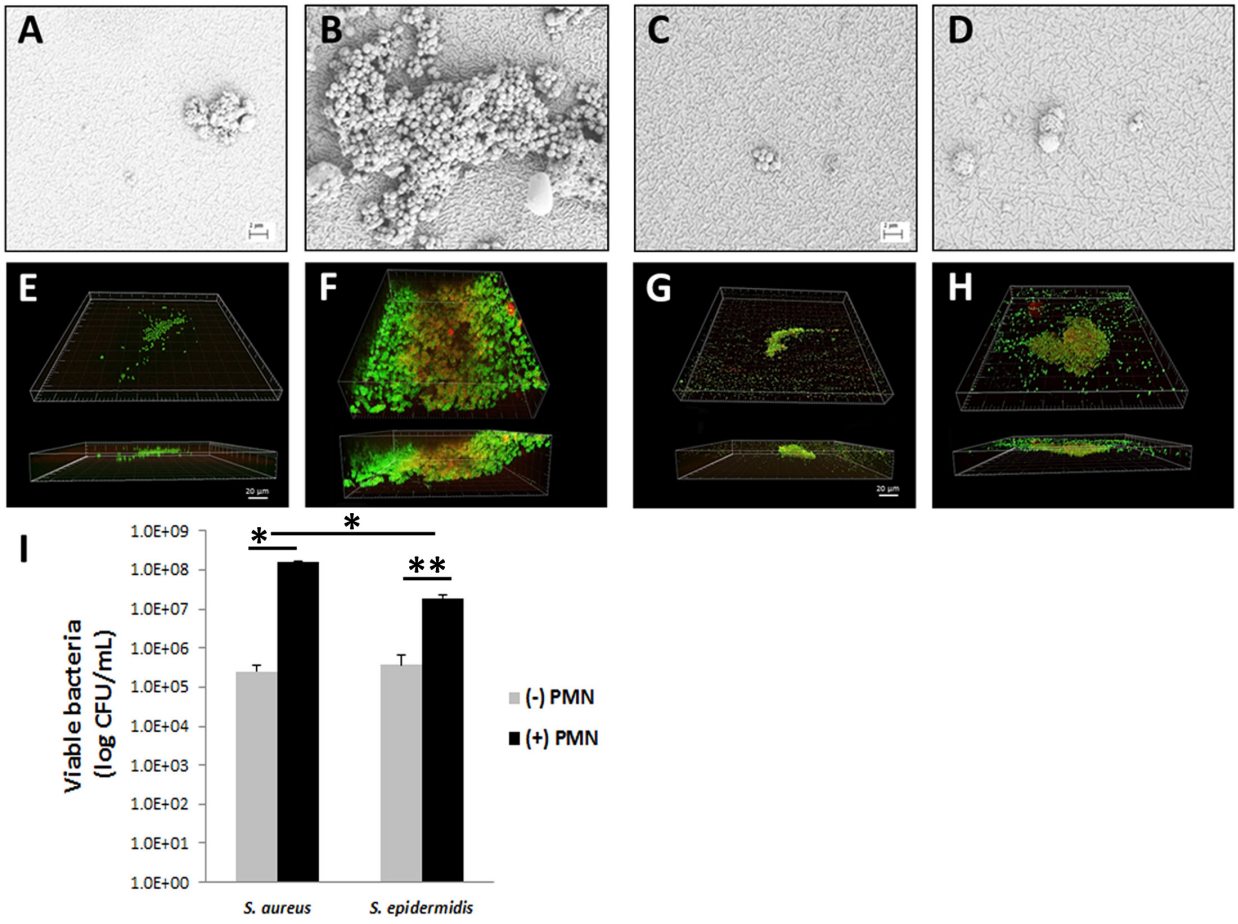

Figure 2. Acceleration of Gram-positive bacterial colonization in the presence of neutrophils. **A**–**D**: Scanning electron microscopy of the colonized bacteria. **A**: *Staphylococcus aureus*, no neutrophils. **B**: *S. aureus* with neutrophils. **C**: *Staphylococcus epidermidis*, no neutrophils. **D**: *S. epidermidis* with neutrophils. Scale bar = 2 µm. **E**–**F**: BacLight staining of the bacteria adherent to the contact lens surfaces. Viable bacteria are shown in green and non-viable bacteria and extracellular DNA in red. **E**: *S. aureus*, no neutrophils. **F**: *S. aureus* with neutrophils. **G**: *S. epidermidis*, no neutrophils. **H**: *S. epidermidis* with neutrophils. Scale bar = 20 µm. **I**: Viable bacteria recovered from the contact lens surfaces. *S. aureus* and *S. epidermidis* showed a statistically significant increase in viable bacteria adherent to the lens surface when coincubated with neutrophils (*$p<0.001$ and **$p = 0.008$ for *S. aureus* and *S. epidermidis*, respectively; two-way ANOVA, $n = 3$). Neutrophil-mediated adhesion was greatest for *S. aureus* (*$p<0.001$, two-way ANOVA, $n = 3$).

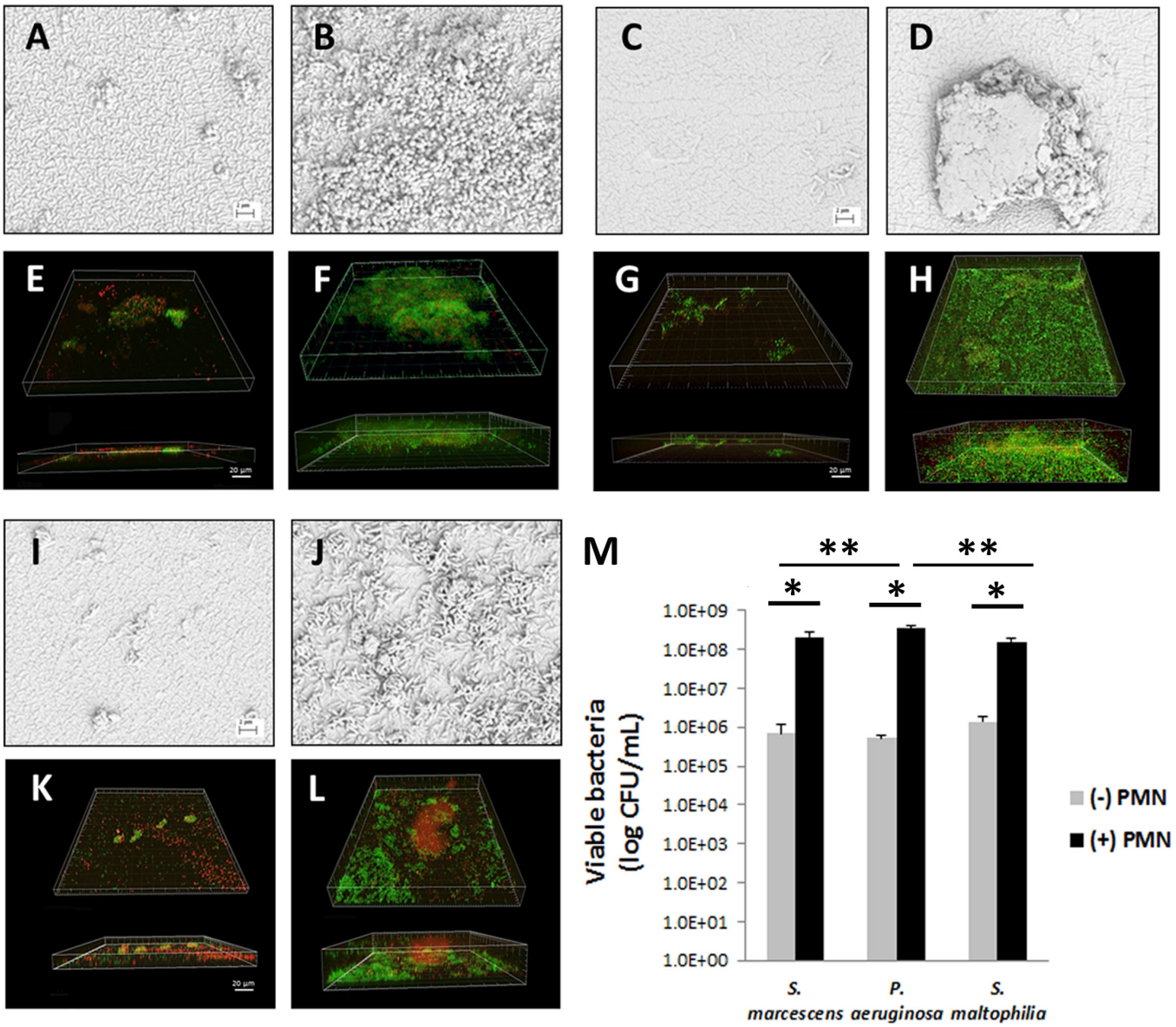

Figure 3. Acceleration of Gram-negative bacterial colonization in the presence of neutrophils. **A**–**D**, **I**–**J**: Scanning electron microscopy of colonized bacteria. **A**: *Serratia marcescens*, no neutrophils. **B**: *S. marcescens* with neutrophils. **C**: *Pseudomonas aeruginosa*, no neutrophils. **D**: *P. aeruginosa* with neutrophils. **I**: *Stenotrophomonas maltophilia*, no neutrophils. **J**: *S. maltophilia* with neutrophils. Scale bar = 2 µm. **E**–**H**, **K**–**L**: BacLight staining of the bacteria adherent to the contact lens surfaces. Viable bacteria are shown in green and non-viable bacteria and extracellular DNA in red. **E**: *S. marcescens*, no neutrophils. **F**: *S. marcescens* with neutrophils. **G**: *P. aeruginosa*, no neutrophils. **H**: *P. aeruginosa* with neutrophils. **K**: *S. maltophilia*, no neutrophils. **L**: *S. maltophilia* with neutrophils. Scale bar = 20 µm. **M**: Viable bacteria recovered from the contact lens surfaces. All three Gram-negative test strains showed a statistically significant increase in viable bacteria adherent to the lens surface when incubated with neutrophils compared to the non-neutrophil control (*p<0.001, two-way ANOVA, n = 3). Bacterial colonization was statistically significantly increased in the presence of neutrophils with *P. aeruginosa* compared with the other two test strains (**p<0.001, two-way ANOVA, n = 3).

For the Gram-positive bacteria, *S. aureus* showed a bumpy topographic appearance that was predominantly viable with sporadic cellular debris intermixed (Figure 4A,B). Non-viable debris could represent extracellular neutrophil DNA or non-viable bacteria. There was a 2.4-fold increase in volume in the presence of neutrophils as measured by cubic microns (p = 0.010, Student *t* test, Figure 4C). *S. epidermidis*, however, showed regional, clumpy areas with and without neutrophils (Figure 4D,E). The difference in the biofilm volume was not statistically significant (p = 0.0573, Student *t* test, Figure

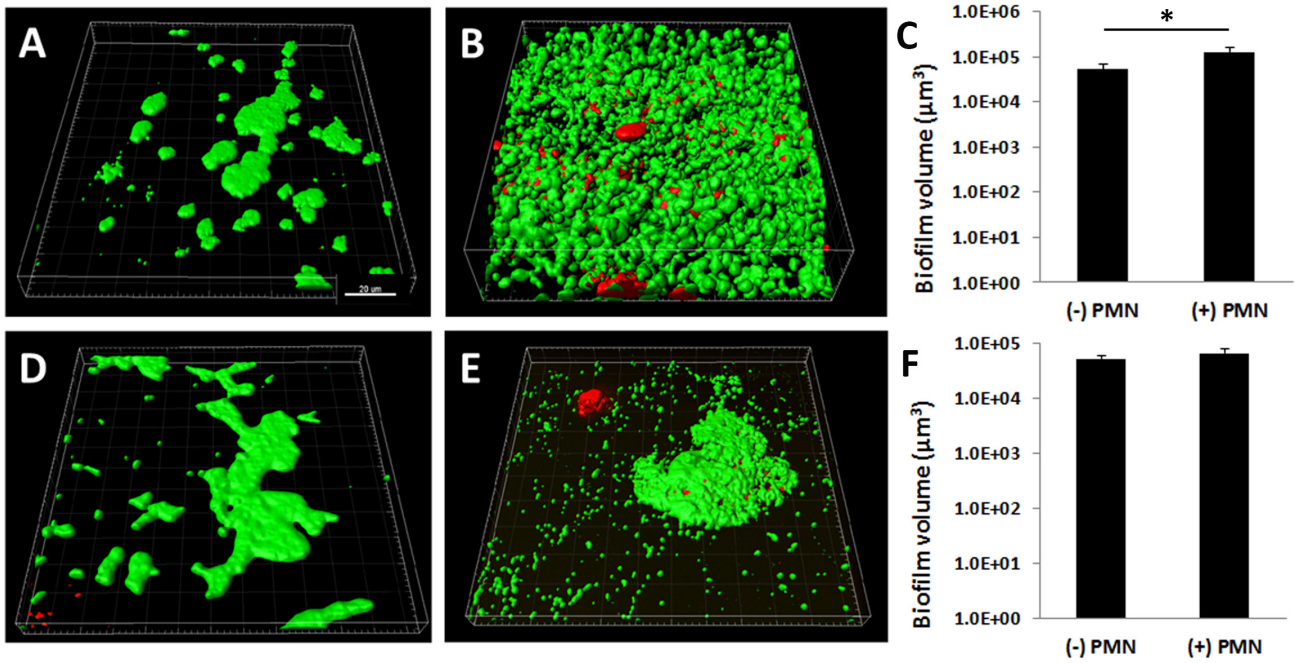

Figure 4. 3D modeling of Gram-positive biofilms. Volumetric reconstruction with the surface function in Imaris software was used to create a three-dimensional (3D) model of colonized bacteria. Green represents viable bacteria. Red represents non-viable bacteria and extracellular DNA. **A**: *Staphylococcus aureus*, no neutrophils. **B**: *S. aureus* with neutrophils. **C**: The biofilm volume was statistically significantly increased in the presence of neutrophils ($p = 0.010$, Student *t* test, $n = 3$). **D**: *Staphylococcus epidermidis*, no neutrophils. **E**: *S. epidermidis* with neutrophils. **F**: There was no statistically significant difference in the biofilm volume between the neutrophils and the non-neutrophil control ($p = 0.0573$, Student *t* test, $n = 3$). Scale bar = 20 µm.

4F). For the Gram-negative test strains, *S. marcescens* and *P. aeruginosa* had the most coalesced areas (Figure 5A,B,D,E). In contrast, *S. maltophilia* showed more of a bumpy topographic appearance (Figure 5G,H). All three Gram-negative bacterial test strains showed statistically significant increases in the biofilm volume ($p<0.001$, $p = 0.008$, and $p = 0.001$, for *S. marcescens*, *P. aeruginosa*, and *S. maltophilia*, respectively; Student *t* test, Figure 5C,F,I).

DISCUSSION

The results of this study demonstrate, for the first time, that multiple genera of bacteria possess the capacity to significantly increase colonization of silicone hydrogel contact lens surfaces in the presence of dying neutrophils. Consistent with our previous observations using invasive *P. aeruginosa* strains 6294 and 6487, this effect was highly significant for *P. aeruginosa* reference strain 9027 [13,16,18]. Although *P. aeruginosa* showed the greatest increase in lens colonization when incubated with neutrophils, *P. aeruginosa* had a higher starting inoculum than the other two Gram-negative strains. Despite this, *P. aeruginosa* colonization of the contact lens surface in the absence of neutrophils did not result in an increase in recovered viable bacteria when compared to *S. marcescens* or *S. maltophilia.* The mechanism(s) driving an increase in *P. aeruginosa* colonization in the presence of neutrophils, either increased adhesion to the lens surface or increased proliferation, is unknown. Given the high recovery rates of *P. aeruginosa* from patients during an infectious event, further studies to investigate these mechanisms are warranted.

A large increase was also observed in the dense 3D architecture and the number of viable organisms recovered from the contact lenses incubated with simulated neutrophils and each of the following bacteria: *S. aureus*, *S. maltophilia*, and *S. marcescens*. Importantly, these bacteria are all pathogens frequently recovered from contact lenses and contact lens storage cases following the development of infectious keratitis or other contact lens–related adverse events. Unlike these test strains, the viability of recovered *S. epidermidis* was increased only slightly when coincubated with neutrophils, and the biofilm volume was not statistically significantly different when compared to bacteria alone. This effect may be strain dependent, as different strains of *S. epidermidis* have been shown to have variable adherence to contact lens

polymers or may be dependent on culture conditions and the specific growth kinetics of this organism [26,27]. Additional studies to tease out these experimental parameters are needed.

Sufficient clinical and epidemiological evidence exists to support a relative risk hierarchy for infectious keratitis arising during contact lens wear [1,28-31]. Soft lenses, whether conventional hydrogel or silicone hydrogel materials, inarguably carry a higher risk for infectious keratitis compared with rigid lenses. One potential contributor to this higher risk lies in the low rate of tear exchange due to the soft lens draping the cornea. In contrast to soft lenses, rigid lenses are readily lifted during blinking to ensure full tear flushing of the underside of the lens. Thus, in the presence of microorganisms that are introduced to the eye as a result of poor hand or case hygiene, any residual bacteria trapped under the soft lens will induce a chronic subclinical inflammatory response that can precipitate neutrophil accumulation with the subsequent establishment of lens-associated bioburden. As used in previous studies, the inclusion of DNase reduces the formation of biofilms and bacterial adhesion to lens surfaces in vitro [14,15]. Together, our data suggest that the presence of substantial neutrophil debris accumulation may drive bacterial colonization under the lens despite the antibacterial properties of the tear film [32-37]. One limitation

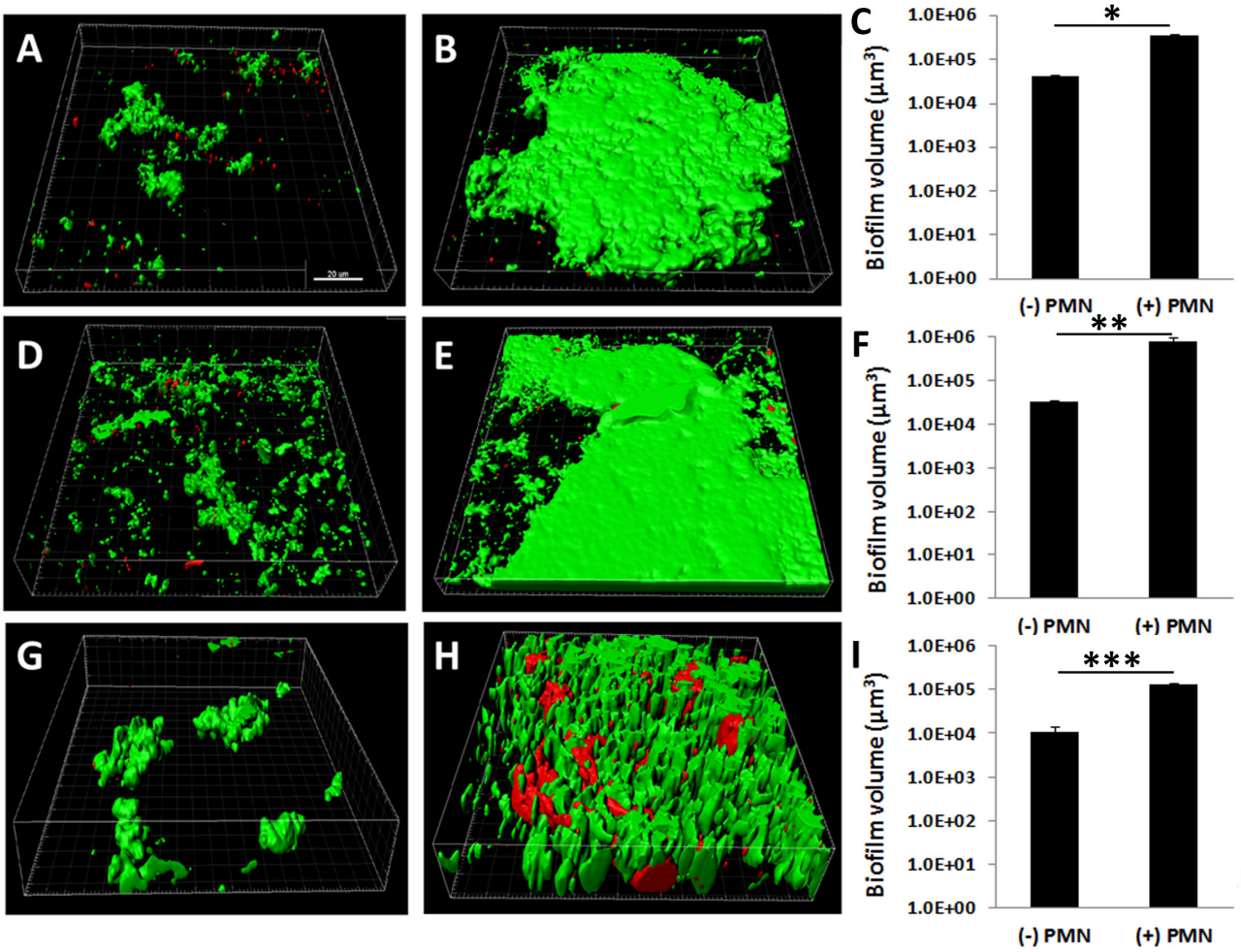

Figure 5. 3D modeling of Gram-negative biofilms. Volumetric reconstruction with the surface function in Imaris software was used to create a three-dimensional (3D) model of colonized bacteria. Green represents viable bacteria. Red represents non-viable bacteria and extracellular DNA. **A**: *Serratia marcescens*, no neutrophils. **B**: *S. marcescens* with neutrophils. **C**: The *S. marcescens* biofilm volume was statistically significantly increased in the presence of neutrophils ($p = 0.001$, Student *t* test, $n = 3$). **D**: *Pseudomonas aeruginosa*, no neutrophils. **E**: *P. aeruginosa* with neutrophils. **F**: The biofilm volume of *P. aeruginosa* was statistically significantly increased in the presence of neutrophils compared to bacteria alone ($p = 0.008$, Student *t* test, $n = 3$). **G**: *Stenotrophomonas maltophilia*, no neutrophils. **H**: *S. maltophilia* with neutrophils. **I**: The biofilm volume of *S. maltophilia* was similarly increased in the presence of neutrophils ($p<0.001$, Student *t* test, $n = 3$). Scale bar = 20 µm.

of this study is that there are clearly substantive differences between the RPMI media used and actual tear fluid. Thus, the results must be interpreted cautiously. However, we have previously shown that the mechanism mediating the formation of neutrophil-enhanced biofilm on contact lens surfaces in vitro was replicated during inoculated contact lens wear in the rabbit eye in vivo [13]. The extent to which the findings reported here correlate with in vivo corneal pathology requires further testing in an animal model.

In the present study, the use of the neutrophil-activating agonist PMA drives the formation of neutrophil extracellular traps (NETs) [38-40]. Extracellular release of cell constituents by the neutrophil during NETosis or necrosis may be a major stimulus for inflammation through regulation of neutrophil activity and stimulation of the respiratory burst [41]. Enzymes released by neutrophils, including myeloperoxidase and neutrophil elastase, can become entrapped within extracellular DNA and in turn, serve as potent neutrophil chemoattractants that promote increased neutrophil recruitment to the site. Thus, during contact lens wear, the stagnation of inflammatory debris derived from host cells under the posterior lens surface may further reflect a maladaptive response of the neutrophil that is unique to the contact lens–wearing eye. It has been reported that neutrophils present on the ocular surface respond differently to various stimuli than neutrophils harvested from peripheral blood [42]. Although we used peripheral blood neutrophils in this study, these data are highly repeatable among multiple different laboratories and have been shown by our laboratory and others to be due to extracellular neutrophil debris and not individual neutrophil response mechanisms [13,15,16]. In addition, data reported by our laboratory and others have shown that necrotic neutrophil debris from repeated freeze–thaw cycles show the same biologic effect as PMA-stimulated neutrophils [16]. Taken together with the absence of any viable neutrophils on the contact lens surface visible by live/dead staining, these data argue in favor of the hypothesis that necrotic or NETosis-related debris contributes to bacterial colonization of the lens.

The capacity of bacteria to adhere to different contact lens polymers has been widely studied and has been reviewed elsewhere [11,43,44]. Due to differences in biophysical properties, silicone hydrogel lenses, on average, tend to bind bacteria in higher numbers than their conventional hydrogel counterparts. This is thought to be a result of differences in the hydrophobicity of the lens polymer and changes that occur to the lens during wear. A recent study by Vijay and colleagues examined the ability of three strains of *S. aureus* and *P. aeruginosa* to adhere to ten different silicone hydrogel lens materials and demonstrated statistically significant differences between lens types and worn compared to unworn lenses [45]. A limitation of the present study is that only one lens material was evaluated and all lenses were unworn making it difficult to extrapolate our findings to other lens types and test conditions. However, the magnitude of the increase in bacterial adherence that we detected was on the order of 2–3 log units when cultured in the presence of neutrophils. We have previously shown a similar response using a conventional hydrogel lens material (etafilcon A) and demonstrated that this response is a result of the charged binding affinities that exist between extracellular debris constituents that facilitate bacterial colonization [13]. Based upon the data reported in our current and previous studies, we hypothesize that polymer differences in adhesion would not statistically significantly impact our findings and that neutrophil accumulation under the lens would override differences in the biophysical parameters of the contact lens itself. This hypothesis is supported by the fact that there is no available epidemiological data to suggest that there are differences in infection rates among the various types of soft contact lens materials [6,46,47]. Additional studies are needed to test this hypothesis, including studies that control for clinical parameters, such as the effects of duration and the modality of lens wear, compliance, and contact lens care solution use.

In a recent laboratory study, Dutta and colleagues reported on the primary factors that may impact bacterial adhesion to contact lenses [43]. These factors included the starting inoculum size, the culture media used, and the length of incubation. Based upon their findings and a summary of the literature investigating bacterial adhesion to contact lens surfaces, the authors concluded that a medium inoculum of 10^6 CFU/ml is most representative of the level of contamination within lens storage cases and represents the optimal inoculum for contact lens bacterial adhesion studies. Although we did not investigate factors such as inoculum size in the present study, we and others have shown that neutrophil-derived cellular debris can dramatically accelerate *P. aeruginosa* colonization even at low inoculum levels, suggesting that this phenomenon is inoculum independent [16,18]. Similar to inoculum, the incubation period in the present study was held constant and evaluated only following an overnight period (18 h) of culture. Because primary adhesion occurs over the first several hours, an investigation into the temporal changes in bacterial adherence and growth kinetics over that 18 h period may provide relevant data about whether bacteria use the neutrophil debris to facilitate the initial attachment phase or support growth of the organism.

In summary, it is currently accepted that adhesion of viable bacteria to the posterior contact lens surface represents

the most likely inciting event in the development of a corneal infiltrative event, either sterile or infectious. These findings suggest that in the setting of chronic inflammation under the lens, multiple genera possess the capacity to utilize inflammatory debris to enhance colonization of the contact lens thus increasing the risk for infectious keratitis.

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